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Assessing the Potential of Hypochlorous Acid-Oxidised Allogeneic Tumour Cells as a Source of Antigen for Dendritic Cell-Based Immunotherapy of Ovarian Carcinoma

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Abstract

Ovarian cancer commonly relapses after remission and activating tumour-specific T cells with dendritic cells loaded with tumour cells is a promising approach to target residual microscopic tumours. SK-OV-3 cells expressing HER-2/neu and MUC1 ovarian antigens were killed and their immunogenicity enhanced by hypochlorous acid (HOCl) oxidation. Treatment with 60 μM HOCl for 1 h induced necrosis in all the cells. Oxidised, but not live SK-OV-3 were engulfed by monocyte-derived DCs. Autologous T cells primed with DCs of HLA-$A2^+$ healthy volunteers loaded with oxidised SK-OV-3 (HLA-$A2^-$) recognised oxidised SK-OV-3 and HLA-A2-restricted epitopes of MUC1 and HER-2/neu. Responses were absent with heat- or hydrochloric acid (HCl)-killed SK-OV-3, thus HOCl oxidation and not cell death/necrosis enhanced the immunogenicity of SK-OV-3. Oxidised SK-OV-3 primed T cells did not respond to oxidised melanoma, or vice versa. Next, T cells of ovarian cancer patients in remission, whose tumours overexpressed MUC1 and/or HER-2/neu, were tested using the same model. DCs of HLA-$A2^+$ and $A2^-$ patients pulsed with oxidised SK-OV-3 stimulated T cells specific for oxidised SK-OV-3, and HER-2/neu and MUC1 epitopes. Oxidised SK-OV-3 loaded DCs further matured with CD40 agonistic antibody or monophosphoryl lipid A, primed stronger $CD4^+$ and $CD8^+$ responses than immature DCs. T cells stimulated this way also recognised autologous tumour cells from ascites, demonstrating that SK-OV-3 shared significant antigenic repertoire with tumours in patients. Finally, this DC-based vaccination was tested *in vivo* using the B16.F10 melanoma model. B16.F10 highly expressed tyrosinase-related protein (TRP)-2, and all the cells were necrotic after 1 h treatment with 60μM HOCl. Mice immunised intravenously and not intraperitoneally or subcutaneously with bone-marrow derived DCs loaded with oxidised B16.F10, responded to TRP-2 and oxidised

B16.F10. Oxidised and not heat- or HCl-killed B16.F10, primed specific T cells *in vivo*. DCs were required for efficient delivery for processing and presentation of oxidised B16.F10 *in vivo*.

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Abbreviations

AFP	Alpha (α)-fetoprotein
AIM-2	Interferon-inducible protein absent in melanoma 2
APCs	Antigen presenting cells
ART-4	Adenocarcinoma antigen recognised by T cells 4
BAGE	B antigen
BCR-ABL	Breakpoint cluster region-Abelson
CAMEL	CTL-recognised antigen on melanoma
CCL	C-C chemokine
CD	Cluster of Differentiation
CDK4	Cyclin-dependent kinase 4
CCR	C-C chemokine receptor
CEA	Carcinoembryonic antigen
CTL	Cytotoxic T lymphocytes
CXCL	C-X-C chemokine
Cyp-B	Cyclophilin B
DC	Dendritic cell
DDB5	Deadbox protein-5
DNA	Deoxyribonucleic acid
ELISPOT	Enzyme-linked immunospot
Ep-CAM	Epithelial cell adhesion molecule
ETV6/AML	Ets variant gene 6/ acute myeloid leukaemia 1 gene ETS
GAGE	G antigen
Gp-100	Glycoprotein 100 kDa
HER-2/neu	Human epidermal receptor 2/ neurological

HEXIM1	Hexamethylene bis-acetamide inducible 1
HLA	Human lymphocyte antigen
HOXB6	Homeobox B6
Hsp	Heat-shock protein
hTERT	Human telomerase reverse transciptase
IFN	Interferon
IL	Interleukin
K-RAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LAGE	L antigen
mAb	Monoclonal antibody
MAGE	Melanoma antigen
MART-1/Melan-A	Melanoma antigen recognised by T cells-1/ melanoma antigen A
MHC	Major histocompatibility complex
MICR	Melanocortin 1 receptor
MUC1, 2	Mucin 1, 2
MUM-1, -2, -3	Melanoma ubiquitous mutated 1, 2, 3
NF-κB	Nuclear factor-kappa B
NPM/ALK	Nucleophosmin/ anaplastic lymphoma kinase fusion protein
N-RAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
NY-ESO-1	New York esophagus 1
p53	Tumour suppressor p53
PBMCs	Peripheral blood mononuclear cells
POLY I:C	Polyinosinic polycytidylic acid
PRAME	Preferentially expressed antigen of melanoma

PSA	Prostate-specific antigen
RNA	Ribonucleic acid
RT	Room temperature
SAGE	Sarcoma antigen
SOX10	Sex determining region Y (SRY)-box 10
SSX-2	Synovial sarcoma, X breakpoint 2
STAT	Signal transducer and activator of transcription
SYT-SSX	Synaptotagmin I/ synovial sarcoma, X fusion protein
TAA	Tumour associated antigen
TEL/AML1	Translocation Ets-family leukaemia/ acute myeloid leukaemia 1
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TOP2A	Topoisomerase (DNA) II alpha 170kDa
TRAG-3	Taxol resistant associated protein 3
TRAIL	Tumour necorsis factor-related apoptosis inducing ligand
Treg	T regluatory cell
TRP-1	Tyrosinase-related protein 1, or gp75
TRP-2	Tyrosinase-related protein 2
TRP-2/INT2	TRP-2/ intron 2
TRP-2/6b	TRP-2/ novel exon 6b
UBQLN1	Ubiquilin 1
VEGF	Vascular endothelial growth factor
WT1	Wilm's tumour gene

Chapter 1

General Introduction

1.1. Introduction: Redefining Cancer Surveillance Theory

In the late 19th century William Coley, a surgeon at the Memorial Sloan-Kettering Cancer Centre in New York observed that cancer patients with infectious episodes often had spontaneous tumour regression (Burnet, 1970). A few years later in 1909, Ehrlich proposed that the immune system was able to control cancer (Ehrlich, 1909). The modern era of tumour immunology really began when Burnet and Thomas (Burnet, 1970; Thomas, 1960) introduced the term 'cancer surveillance' in the 1950s, proposing that the immune system of immunocompetent hosts continually recognised and eliminated tumour cells via their expression of tumour-associated antigens (TAAs). This hypothesis was disputed in the 1980s on the basis of studies showing tumours failed to develop more rapidly in nude mice (which lack T and B cells but not natural killer [NK] cells) than in wild-type mice. However in the 1990s, compelling evidence indicated that immunodeficient mice were at greater risk for spontaneous tumour development of some tumours. More recently, it has become clear that whilst the adaptive immune system can actively eliminate tumour cells and prevent the formation of neoplasia, inflammation can also facilitate tumour progression in part by sculpturing the immunigenicity of tumour as it develops. The cancer immunosurveillance hypothesis has therefore been refined into one termed "cancer immunoediting". The 3 Es is being used to describe cancer immunoediting – elimination (i.e. cancer surveillance), equilibrium, and escape. During elimination, the immune system attempts to eradicate the cancer. If this fails, the cancer and the immune system achieve an equilibrium in which the immune system is able to contain but not eliminate the cancer. In the equilibrium phase, the cancer is under constant pressure from the immune system but can also undergo genetic changes that can lead to increased immune resistance. If this occurs, the cancer cells can become resistant

and escape from immune attack even in the presence of an intact immune system. Understanding the intricate balance between cancer elimination and evasion will be invaluable in tilting immunotherapy in favour of the host. This introduction discusses the concept of cancer immunoediting and summarised the recent progressed in cancer vaccines development. This Ph.D. project focused mainly on ovarian carcinoma and the major immunological aspects of this cancer are discussed in detail. A smaller section on melanoma is also presented. Lastly, the potential use of hypochlorous acid in tumour immunotherapy is discussed.

1.2. The Immunological Control of Cancer

1.2.1 Evidence of cancer surveillance

Three lines of evidence strongly suggest that cancer surveillance occurs in humans. Firstly, immunosuppressed transplant recipients usually display a higher incidence of viral and non-viral cancers than age-matched immunocompetent control populations. Assessment of 5692 renal transplant patients from 1964 to 1982 in Scandinavia revealed increased incidences of colon, lung, bladder, kidney, ureter and endocrine tumours (Birkeland *et al.*, 1995). Also, 925 patients who received cadaveric renal transplants from 1965 to 1998 in Australia and New Zealand showed higher frequencies of a variety of non-viral cancers than the general population (Sheil, 1986). These observations suggested that immunosuppression intervention predisposed transplant patients either to *de novo* tumour formation or allowed the outgrowth of occult tumours which had previously been contained by a functioning immune system. Secondly, cancer patients can develop spontaneous adaptive and innate immune responses to the tumours that they bear (Old, 1981; Old and Chen, 1998; Sahin *et al.*, 1995). Thirdly, the presence of $CD8^+$ lymphocytes in the tumour can

indicate a better prognosis and enhanced patient survival in colon cancer (Naito *et al.*, 1998; Strater *et al.*, 2005), oesophageal cancer (Schumacher *et al.*, 2001; Yasunaga *et al.*, 2000), cervical cancer (Piersma *et al.*, 2007), ovarian cancer (Sato *et al.*, 2005) and melanoma (Haanen *et al.*, 2006).

Technological advancements in mouse genetics and monoclonal antibody (mAb) production have helped to elucidate the cellular basis of cancer surveillance. In particular, gene-targeted mice lacking the recombinase activating gene (RAG)-2 (Shankaran *et al.*, 2001) allowed the generation of mice that lack peripheral αβ T cells, γδ T cells, B cells or NK cells with which to study the influence of host lymphocyte deficiency on tumour development. Studies showed that 129/SvEvRAG$^{-/-}$ and C57BL/6 RAG$^{-/-}$ mice receiving subcutaneous injection of chemical carcinogen 3'-methylcholanthrene (MCA) developed sarcomas at a faster and higher rate than wild-type controls (Shankaran *et al.*, 2001; Smyth *et al.*, 2000). Also, *Helicobacter* negative RAG$^{-/-}$ 129/SvEv mice maintained in pathogen-free mouse facility and broad-spectrum antibiotics had more spontaneous epithelial tumours than the wild-type (Shankaran *et al.*, 2001). T, NK and NKT cells are involved in tumour defence as depleting NK and NKT cells with anti-NK1.1 mAb or anti-asialo-GM1 in C57BL/6 mice resulted in more tumours than wild-type (Hayakawa *et al.*, 2003; Smyth *et al.*, 2000; Smyth *et al.*, 2001). Also, mice without αβ or γδ T cells were more susceptible to MCA-induced tumour formation than wild-type (Girardi *et al.*, 2001).

The role of γδ T cells in anti-tumour immunity was highlighted in a recent phase I clinical trial where patients with metastatic hormone-refractory prostate cancer were treated with γδ T cell agonist zoledronate, either alone or in combination with low-

dose IL-2 to activate peripheral blood γδ T cells (Dieli *et al.*, 2007). Most of the nine patients who were treated with zoledronate and IL-2 had peripheral γδ T cells that produced IFN-γ and perforin. These patients also maintained serum levels of TRAIL. Furthermore the numbers of these γδ cells correlated positively with declining prostate-specific antigen levels and objective clinical outcomes that comprised three instances of partial remission and five of stable disease. By contrast, most patients treated only with zoledronate failed to sustain either γδ T cell numbers or serum TRAIL, and showed progressive clinical deterioration. Though αβ T cells are implicated in anti-tumour surveillance, a study involving a two-stage chemical carcinogenesis protocol of single exposure to the mutagen 7,12-dimethylbenz[a]anthracene [DMBA] followed by repeated applications of the inflammation-inducing phorphol ester, phorbol 12-myristate 13-acetate [PMA] to induce malignant lesions, demonstrated that αβ T cell deficiency was associated with reduced tumour development and progression (Girardi *et al.*, 2003). These novel tumour-promoting T cells (T-pro cells) were $\alpha\beta^+$ $CD8^+$ $CD44^+$ $CD62^-$ and produced substantial amounts of IFN-γ and TNF-α, but little perforin (Roberts *et al.*, 2007). They shared intriguing similarities with uterine NK cells whereby they were not cytolytic and they produced cytokines associated with trophoblast growth and invasion of the uterine wall (Ashkar *et al.*, 2000).

Studies showed that IFN-γ protected the host against tumour growth (Dighe *et al.*, 1994; Kaplan *et al.*, 1998; Shankaran *et al.*, 2001; Street *et al.*, 2001; Street *et al.*, 2002), and injection of IFN-γ neutralising mAbs abolished this protection (Dighe *et al.*, 1994). Moreover, 129/SvEv mice lacking IFN-γ responsiveness ($IFNGR1^{-/-}$ or

STAT1$^{-/-}$ mice) had more tumours than wild-type mice (Shankaran *et al.*, 2001). IFN-α/β is also implicated in preventing cellular transformation by enhancing the p53 tumour-suppressor gene expression in cells (Takaoka *et al.*, 2003). In addition, perforin (pfp) and tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) are involved in tumour cytotoxicity (Smyth *et al.*, 2000; Smyth *et al.*, 2003; Street *et al.*, 2001; van den Broek *et al.*, 1996). The NKG2D, an activating receptor expressed on all NK cells, some αβ and γδ T cells, and some NKT cells, is constitutively expressed on some tumour cell lines (Coudert and Held, 2006; Hayakawa *et al.*, 2003; Pardoll, 2003; Pende *et al.*, 2002) and might play a role in eliminating transformed cells. Its ligands include MHC class I chain-related proteins A and B (MICA/B) which are often expressed on primary carcinomas of the lungs, kidneys, ovary, prostate, colon (Groh *et al.*, 1999) and melanoma (Vetter *et al.*, 2002), and UL16 binding proteins (Cosman *et al.*, 2001; Pende *et al.*, 2002). Conversely, the sustained expression of MICA by tumours can also elicit NKG2D downregulation, perhaps indicating 'immunoevasion' (see section 1.2.3). In a study, it was observed that the constitutive Rae-1 [retinoic acid early inducible 1; expressed in mouse and a ligand for NKG2D] epsilon transgene expression in normal epithelium caused the downregulation of local and systemic NKG2D expression, which in turn led to generalised but reversible defects in NK cell-mediated cytotoxicity and mild $CD8^+$ T cell defects (Oppenheim *et al.*, 2005). This downregulation of NKG2D correlated positively with increased incidence and progression of cutaneous carcinogenesis, thus emphasising the role of NKG2D in tumour surveillance.

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1.2.2. Equilibrium phase

Based on the evidence of cancer surveillance, it could be envisaged that a significant percentage of transformed cells are eliminated during this phase. However, some tumour cells might withstand the formidable pressure exerted by the cancer surveillance's arsenal and emerge into clinically detectable malignant disease. Dunn and colleagues (Dunn *et al.*, 2004a; Dunn *et al.*, 2004b) suggested the existence of a period of tumour dormancy or equilibrium phase, whereby the immune system could initially inhibit the growth of heterogenous tumours that were composed of rapidly mutating cells but as the original tumour cells are destroyed, new variants arise that make them more resistant to immune attack. This equilibrium phase could persist for years and could allow the selection of tumour cells with reduced immunogenicity.

Studies were done to compare the immunogenicities of tumours derived from immunocompromised and immunocompetent mice. It was found that tumours derived from wild-type 129/SvEv mice grew progressively in both strain-matched wild-type and $RAG^{-/-}$ 129/SvEv mice, while 8 out of 20 tumours from $RAG^{-/-}$ 129/SvEv mice were rejected in wild-type mice (Shankaran *et al.*, 2001). Also, MCA-induced sarcoma from nude (Svane *et al.*, 1996) or severe combined immunodeficiency [SCID] mice (Engel *et al.*, 1997) were rejected more frequently in wild-type after transplantation than those derived from wild-type. Lymphomas from $pfp^{-/-}$ mice grew avidly in $pfp^{-/-}$ recipients but were rejected in the wild-type controls (Street *et al.*, 2001), suggesting that tumours growing in immunocompromised environment are more immunogenic than tumours developing in immunocompetent hosts.

A clinical scenario that demonstrated the existence of an equilibrium phase in the humans is the transmission of cancer from organ transplant donors to recipients. One study described two patients developing melanoma 1 to 2 years after receiving a kidney transplant each from the same donor, who was considered tumour-free having been treated for melanoma 16 years earlier (MacKie *et al.*, 2003). Similar observations were made in recipients of allografts from those considered as healthy donors (Elder *et al.*, 1997; Penn, 1991; Penn, 1995; Penn, 1996; Suranyi *et al.*, 1998), suggesting that tumours had been kept in equilibrium in the donor and continuously administrating immunosuppressive drugs might facilitate the growth of occult cancer. Other clinical evidence also supports the existence of the equilibrium phase. First, the existence of an immune response to premalignant monoclonal gammopathy of undetermined significance (MGUS) cells that eventually progress to multiple myeloma (MM) is consistent with the equilibrium phase, with the immune system controlling but not eliminating MGUS cells that eventually evolve and progress to malignancy (Dhodapkar *et al.*, 2003). Second, passive immunisation with idiotypespecific antibody in conjunction with either cytokine therapy or chemotherapy, can induce remission in some patients with low-grade B cell lymphoma; however, tumour cells are not completely eliminated and can be detected in the blood or bone marrow for up to 8 years following clinical remission (Davis *et al.*, 1998). Studies in pediatric acute myeloid leukemia patients treated with either chemotherapy or chemotherapy combined with autologous bone marrow trans-plantation also suggested a role of the immune system in maintaining equilibrium and establishing long-term remission (Montagna *et al.*, 2006). It has also been noted that tumors can remain dormant in patients for many years, but relapse after long periods (at least 10 years) of tumour remission (Callaway and Briggs, 1989; Demicheli *et al.*,

1996; Stewart *et al.*, 1991), hence suggesting the possibility of immune control with subsequent escape in these cases.

1.2.3. Mechanisms of tumour evasion of immune system

Tumour cells that survived the equilibrium phase often possess several mechanisms to evade the immune system (Rabinovich *et al.*, 2007). Many human tumours lose their HLA class I proteins (Algarra *et al.*, 2000; Marincola *et al.*, 2000) or have deficient antigen processing and presentation pathways such as transporter associated with antigen processing-1 and the immuno-proteasome subunits low molecular mass protease [LMP] 2 and 7 (Seliger *et al.*, 2000) to avoid immune detection. Also, several human lung carcinoma cell lines were unresponsive to IFN-γ due to absence or abnormal IFN-γ receptor signalling (Kaplan *et al.*, 1998) and hence failed to upregulate MHC class I pathway activity when exposed to IFN-γ. Tumours could secrete soluble factors, such as vascular endothelial growth factor [VEGF] to suppress DC differentiation and maturation (Dikov *et al.*, 2001), soluble Fas ligand to induce lymphocyte apoptosis, or soluble MICA products to inhibit NKG2D-mediated killing by immune cells (Groh *et al.*, 2002; Ryan *et al.*, 2005). In addition, IL-10, TGF-β (Li *et al.*, 2006) and prostaglandin-E [PGE_2] (He and Stuart, 1999) released by tumour cells could inhibit immune responses of DCs and T cells. Galectin-1 (Rubinstein *et al.*, 2006) and indoleamine 2,3-dioxygenase [IDO] (Uyttenhove *et al.*, 2003) also inhibit T cell activation.

Immunosuppressive T cell populations in tumours, such IL-13 producing NKT cells (Terabe *et al.*, 2005) and $CD4^+$ $CD25^+$ T regulatory cells [Tregs] (Sakaguchi *et al.*, 2001) have been shown to suppress anti-tumour responses. Two studies demonstrated

that depleting $CD4^+$ $CD25^+$ Tregs by anti-CD25 mAb enabled mice to reject tumours (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999). Specifically, elevated levels of $CD4^+$ $CD25^+$ Tregs were detected in patients with non-small cell lung cancer, and cancer of the ovary (Woo *et al.*, 2001), breast and pancreas (Liyanage *et al.*, 2002) and correlated with poor prognosis and survival (Curiel *et al.*, 2004; Zou, 2006) [see sections 1.3.4 and 1.4.1 for further discussions on Tregs]. Tumour infiltrating lymphocytes [TILs] in melanoma (Zea *et al.*, 1995), renal cell cancer (Cardi *et al.*, 1997), ovarian cancer (Lai *et al.*, 1996) and oral cancer (Reichert *et al.*, 1998) have also been found to lose their signal transducer CD3-ζ chain (CD3-ζ) that prevented their activation. In a recent study involving patients with breast cancer, it was found that the number of $CD4^+$ and $CD8^+$ T cells expressing the IL-7 receptor was reduced in the peripheral blood of these patients (Vudattu *et al.*, 2007). These T cells did not respond to IL-7 stimulation despite expressing IL-7Rα. Further analysis showed that these T cells had decreased IL-2 and IFN-γ production when stimulated with phorbol 12-myristate 13-acetate (PMA)/Ionomycin, and hence suggested a defect in the IL-7 signaling pathway.

1.2.4. Inflammation and cancer

Clinical and epidemiologic studies have suggested a strong association between chronic infection, inflammation, and cancer (Coussens and Werb, 2002; Dobrovolskaia and Kozlov, 2005; Fox and Wang, 2007; Hussain *et al.*, 2003). For example, liver cancer is associated with excessive alcohol consumption and infection with Hepatitis B and C virus, colon cancer is associated with inflammatory bowel disease, and gastric cancer can develop in individuals chronically infected with *Helicobacter pylori*. The NF-κB kinase/ NF-κB (IKK/NF-κB) signaling pathway

provides a key molecular link between inflammation and malignant transformation. It is activated by many proinflammatory cytokines (Karin, 2006; Karin and Greten, 2005), and following recognition of pathogen-associated molecular patterns by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain-like (NOD-like) receptors. Crohn's disease which is associated with mutations in the NOD2 locus and enhanced IL-1β production (Eckmann and Karin, 2005; Hugot *et al.*, 2001) might predispose the individuals to colorectal cancer (Kurzawski *et al.*, 2004).

Several proinflammatory cytokines can promote tumour development. Prolonged TNF-α production can induce NF-κB–dependent expression of anti-apoptotic and proliferative genes, enhance angiogenesis, and evade immune surveillance by conferring resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Elgert *et al.*, 1998). IL-6, induced through TLR activation or produced endogenously (e.g. by stromal cells), can promote cell cycle progression and exacerbate the effects of TNF-α. Indeed, IL-6 has been implicated in the development of multiple myeloma (Bommert *et al.*, 2006), Kaposi's sarcoma (Li *et al.*, 2001; Osborne *et al.*, 1999) and Hodgkin's lymphoma (Cozen *et al.*, 2004). IL-10 and TGF-β can exhibit tumour-suppressing activity under certain circumstances; mice lacking either IL-10 or TGF-β1 are more susceptible to cancer in response to infection with enteric bacteria (Berg *et al.*, 1996; Engle *et al.*, 2002; Sellon *et al.*, 1998). In contrast, they can also promote tumour development by inhibiting DC activation, suppress T cell responses, block the proinflammatory effects of macrphages, NK cells, and neutrophils, and facilitate the induction of Tregs (Doganci *et al.*, 2005; Ghiringhelli *et al.*, 2005; Kortylewski *et al.*, 2005; Liu *et al.*, 2007; Yu *et al.*, 2007). IL-10, IL-6, and

VEGF can activate STAT3 in tumour cells to endow them with enhanced survival, metastatic, and angiogenic properties (Yu *et al.*, 2007). STAT3 can also down-regulate molecules necessary for anti-tumour immune responses (e.g., IL-12, IFN-γ, and IFN-β), which are also produced following NF-κB activation. Hence STAT3 can block the ability of NF-κB to stimulate anti-tumour immunity (Hoentjen *et al.*, 2005; Schottelius *et al.*, 1999) but cooperate with it to promote oncogenesis (Yu *et al.*, 2007). Small-molecule inhibitors targeting STAT3 directly or indirectly can block the signalling effects of cytokines such as IL-6 and TGF-β and reverse the abnormal differentiation of DCs in cancer (Kortylewski *et al.*, 2005; Nefedova *et al.*, 2004).

IL-23, which belongs to the IL-12 family of proinflammatory heterodimeric cytokines, has been shown to promote tumour growth by upregulating metalloproteinase (MMP) 9, increasing angiogenesis, and decreased recruitment of $CD8^+$ T cells to tumours (Langowski *et al.*, 2006). However, in other studies, mice inoculated with IL-23-transduced tumour cells displayed anti-tumour properties (Lo *et al.*, 2003; Ugai *et al.*, 2003). IL-17 is an important inflammatory cytokine (Mangan *et al.*, 2006) that is produced via STAT3 activation triggered by IL-23 (Cho *et al.*, 2006). It might be important for localising and amplifying inflammation as its production also leads to the induction of many proinflammatory factors, including TNF-α, IL-6, and IL-1β (Langrish *et al.*, 2005; Park *et al.*, 2005; Ruddy *et al.*, 2004). In primary non-small cell lung carcinoma samples, IL-17 expression was frequently detected in TILs and was associated with increased tumour vascularity (Numasaki *et al.*, 2005). Also, IL-17 might act with IL-6 to promote tumour development as the enhanced cervical cancer growth elicited by IL-17 was associated with increased expression of IL-6 and macrophage recruitment to the tumour sites (Tartour *et al.*,

1999). Similar to IL-23, there is also evidence of IL-17 playing a role in tumour surveillance in immunocompetent mice (Benchetrit *et al.*, 2002).

1.3. Recent Progress in Developing Effective Cancer Vaccines

1.3.1. Identifying tumour-associated antigens

Since the cloning of *MAGE-1* (van der Bruggen *et al.*, 1991), the first gene reported to encode a human tumour antigen recognised by T cells, molecular identification and characterisation of novel tumour-associated antigens (TAAs) has rapidly evolved. This has been fuelled in part by the advancement of new technologies, such as using a) specialist software to predict epitopes of T cells on the basis of known HLA-binding motifs, b) biochemical methods (e.g. chromatography, mass spectrometry) to elute and fractionate TAA peptides from tumour cells, c) DNA microarray technology (e.g. representational difference analysis [RDA], differential display [DD], suppression subtractive hybridisation [SSH], and serial analysis of gene expression [SAGE]) to compare gene expression profiles of tumour to normal tissues. In addition, a large collection of TAAs have been identified using antbodies by serological expression of cDNA expression libraries or SEREX technology (i.e. screening of complementary DNA expression libraries with serum from cancer patients), and can be found in the database of the Institute for Cancer Research (http://www.licr.org/SEREX.html). TAAs recognised by T cells are summarised here.

TAAs recognised by T cells can be broadly classified according to their tissue distribution and HLA-allele (or MHC) restriction. MAGE-1, the first TAA to be identified, belongs to the cancer-testis (CT) antigens family. The CT antigens are so-named due to their expression in various types of cancers (including ovarian

carcinoma and melanoma) and restricted expression in normal spermatocytes and/ or spermatogonia of testis, and occasionally in placenta. Other CT antigens include other MAGEs (Lucas *et al.*, 1999; Lucas *et al.*, 2000), the BAGE (Boel *et al.*, 1995) and GAGE (Van den Eynde *et al.*, 1995) families, NY-ESO-1 and its alternative open-reading frame (ORF) products (LAGE, CAMEL) (Aarnoudse *et al.*, 1999; Jager *et al.*, 1998; Lethé *et al.*, 1998), SAGE (Martelange *et al.*, 2000), SSX-2 (Ayyoub *et al.*, 2002) and TRAG-3 (Zhu *et al.*, 2003). NY-ESO-1 is also expressed in normal ovary. Both MHC Class I HLA- and Class II HLA-restricted epitopes of CT antigens have been identified (Chaux *et al.*, 1999a; Chaux *et al.*, 1999b; Consogno *et al.*, 2003; Manici *et al.*, 1999; Slager *et al.*, 2003; Tatsumi *et al.*, 2003; Wang *et al.*, 2004; Zeng *et al.*, 2000). CT antigens result from re-activation of genes which are normally silent in adult tissues but are transcriptionally activated in different tumour histotypes. They represent attractive targets for cancer vaccines because the very low level of class I and II HLA molecules in the testis, placenta and ovary protect tissues from acting as targets for autoimmune reactions (Hutter and Dohr, 1998; Jassim *et al.*, 1989).

Differentiation antigens such as CEA (Tsang *et al.*, 1995), Ep-CAM (Nagorsen *et al.*, 2000), MC1R (Salazar-Onfray *et al.*, 1997), gp-100 (Barker et al., 1995; Kawakami *et al.*, 1995; Kawakami *et al.*, 1998; Skipper *et al.*, 1996b; Tsai *et al.*, 1997), MART-1/Melan-A (Coulie *et al.*, 1994; Kawakami *et al.*, 1994a; Romero *et al.*, 1997; Schneider et al., 1998), PSA (Corman *et al.*, 1998; Correale *et al.*, 1997) and tyrosinase (Benlalam *et al.*, 2003; Brichard *et al.*, 1996; Kang *et al.*, 1995; Kawakami *et al.*, 1995b; Kittlesen *et al.*, 1998; Wölfel *et al.*, 1994) are TAAs that are only expressed in normal and neoplastic cells of a defined lineage. MHC Class II HLA-restricted epitopes have also been found for gp-100 (Kierstead *et al.*, 2001; Lapointe

et al., 2001; Li *et al.*, 1998), MART-1/Melan-A (Zarour *et al.*, 2000), PSA (Corman *et al.*, 1998) and tyrosinase (Kierstead *et al.*, 2001; Kobayashi *et al.*, 1998a; Kobayashi *et al.*, 1998b; Topalian *et al.*, 1994; Topalian *et al.*, 1996). This group of TAAs represents self antigens that are commonly used in current clinical vaccination trials with CT antigens. The third type of TAAs consists of overexpressed self antigens which occur in a range of different types of tumours. Some examples are Adipophilin (Schmidt *et al.*, 2004), AIM-2 (Harada *et al.*, 2001), AFP (Butterfield *et al.*, 1999), ART-4 (Kawano *et al.*, 2000), Cyp-B (Gomi *et al.*, 1999), HER-2/neu (Fisk *et al.*, 1995; Kawashima *et al.*, 1999; Okugawa *et al.*, 2000; Peoples *et al.*, 1995; Rongcun *et al.*, 1999), hTERT (Minev *et al.*, 2000; Vonderheide *et al.*, 2001), MUC1 (Brossart *et al.*, 1999; Domenech *et al.*, 1995), MUC2 (Böhm *et al.*, 1998), PRAME (Ikeda *et al.*, 1997; Kessler *et al.*, 2001), SOX10 (Khong and Rosenberg, 2002b) and WT1 (Azuma *et al.*, 2002; Ohminami *et al.*, 2000; Oka *et al.*, 2000). TAAs of the apoptotic proteins such as livin and surviving, (Andersen *et al.*, 2000; Andersen *et al.*, 2001; Casati *et al.*, 2003; Schmidt *et al.*, 2003; Schmitz *et al.*, 2000; Schmollinger *et al.*, 2003) and tumour suppressor proteins [e.g. p53] (Azuma *et al.*, 2003; Umano *et al.*, 2001) also belong to this group. These TAAs can result from the expression of novel epitopes from widely expressed proteins as a result of splicing aberrations, alternative ORFs or post-translational splicing. As they are expressed in normal tissues at a lower level, they are likely to make weak tumour-rejection antigens as they might trigger some degree of tolerance. This could be overcome by using potent vaccination protocols to activate and expand the remaining low-avidity T cells (see following sections).

Point mutations of normal genes can occur during neoplastic transformation and/or progression to give rise to unique TAAs (e.g. β-catenin (Robbins *et al.*, 1996), CDK4

(Wölfel *et al.*, 1995), caspase-8 (Mandruzzato *et al.*, 1997), Hsp70-2 M (Gaudin *et al.*, 1999), MART-2 (Kawakami *et al.*, 2001), MUM-1 (Coulie *et al.*, 1995), MUM-2 (Chiari *et al.*, 1999), MUM-3 (Baurain *et al.*, 2000), Myosin (Zorn and Hercend, 1999b). In mouse models, such unique TAAs are responsible for the rejection of tumour transplants in mice and have been defined as tumour-specific transplantation antigens [TSTA] (Dudley and Roopenian, 1996). In cancer patients, response to the unique TAAs appears to be associated with a good prognosis (Baurain *et al.*, 2000; Karanikas *et al.*, 2001; Novellino *et al.*, 2003). However, the major drawback is they are only useful in single patient-tailored anti-tumour immunotherapies. A few such altered antigen epitopes which are specific to tumours and are found in several different tumours, are generated from splicing aberrations [e.g. TRP-2/INT2 and TRP-2/6b] (Khong and Rosenberg, 2002a; Lupetti *et al.*, 1998) or common point mutations [e.g. N-RAS and K-RAS] (Gjertsen *et al.*, 1997; Linard *et al.*, 2002) as part of the neoplastic development. In several malignancies, particularly in leukaemia, translocation of chromosomes occurs to which results in fusion of distant genes. This gives rise to fusion proteins characteristic to each type of disease [e.g. BCR-ABL in chronic myelogenous leukaemia (CML) (Norbury *et al.*, 2000; Wagner *et al.*, 2003; Yotnda *et al.*, 1998b), DEK-CAN and TEL/AML1 in acute myelogenous leukaemia (AML) (Ohminami *et al.*, 1999; Yun *et al.*, 1999), ETV6/AML (Yotnda *et al.*, 1998a) and NPM/ALK in acute lymphocytic leukemia (ALL) (Passoni *et al.*, 2002), and SYT-SSX in synovial sarcomas (Worley *et al.*, 2001)]. These fusion proteins can generate new $CD8^+$ and $CD4^+$ T cell epitopes spanning the fusion junction.

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1.3.2. Delivering antigens: dendritic cells as natural adjuvants

Dendritic cells are the key regulators of all immune responses (Banchereau *et al.*, 2000; Guermonprez *et al.*, 2002; Heath and Carbone, 2001). They comprise of several subsets that are located in both lymphoid and non-lymphoid tissues. Three main DC subsets have been identified based on their cell surface markers and functional properties: conventional monocyte-derived DCs, plasmacytoid DCs (pDCS) and follicular DCs (FDCs). The monocyte-derived DCs (referred to here simply as DCs) are widely used in human clinical studies and are discussed below. DCs are responsible for inducing cellular immunity to pathogens by priming T cells into effector and memory cells, and activating NK and NKT cells (Fernandez *et al.*, 1999; Kadowaki *et al.*, 2001). They also induce humoural immunity by activating naïve and memory B cells (Caux *et al.*, 1997; Jego *et al.*, 2003). In addition, DCs are responsible for processing and presenting tumour antigens to naïve $CD8^+$ and $CD4^+$ T cells, and to activate them into CTLs and effector T helper cells, respectively, to combat tumour (Berzofsky *et al.*, 2004; Gilboa, 2004; Pardoll, 1998).

Given the potent immunostimulatory capacity of DCs, one of the major questions in developing effective DC vaccine protocols is the method of delivery of tumour antigens to the immune system via DCs. There are two general approaches, i.e. the *in vivo* route and the *ex vivo* route. The *in vivo* route involves the injection of antigen mixed with adjuvant into a patient. This method is relatively simple and abolishes the laborious task of culturing and loading DCs with tumour antigens *ex vivo*. An example is to deliver DNA encoding antigens (Garcia-Hernandez *et al.*, 2007; Porgador *et al.*, 1998) by gene gun to the resident immature DCs in the skin. Another example is using anti-DEC-205 antibody to target tumour antigens to DC via their

DEC-205 receptors (Bonifaz *et al.*, 2004; Jiang *et al.*, 1995). For example, injecting mice with anti-DEC 205 antibodies coupled to tyrosinase-related protein-2 (TRP-2) and gp-100 led to the induction of anti-tumour responses and eradication of tumour in 70% of the mice (Mahnke *et al.*, 2005). Methods to simultaneously target and activate DCs *in vivo* using antigens fused to chemokines (Biragyn *et al.*, 2001) and immunoglobulin G (IgG) Fc fragment (You *et al.*, 2001) have also been described.

The alternative and more popular approach is to generate and load autologous DCs *ex vivo* (Banchereau and Palucka, 2005; Fong and Engleman, 2000; Gilboa, 2007). The key advantage is that DCs can be matured and differentiated *ex vivo* into potent antigen presenting cells (Kryczek *et al.*, 2006a), and upon administration can efficiently migrate to draining lymph nodes and respond appropriately to local stimuli hence circumvent the problems encountered with *in vivo* vaccination. Also, the high endocytic capacity of DCs can be exploited *ex vivo* by loading them with antigens of choice, whether peptides or proteins derived from TAAs, whole allogeneic or autologous tumour cell lysates, exosomes, necrotic or apoptotic tumour cells, tumour RNA or DNA. Indeed, a degree of success has been observed in several clinical trials using this approach (Banchereau *et al.*, 2001; Hersey *et al.*, 2004; Nestle *et al.*, 1998; Rosenberg *et al.*, 2004; Schuler-Thurner *et al.*, 2002; Thurner *et al.*, 1999). In addition, DCs can be genetically modified to increase their secretion of IL-12, granulocyte-monocyte colony stimulating factor (GM-CSF) (Nakamura *et al.*, 2002; Ojima *et al.*, 2007; Vegh and Mazumder, 2003), anti-apoptotic proteins (Medema *et al.*, 2001), or fused with tumour cells to produce fusion cells that express both DC and tumour markers (Avigan *et al.*, 2004).

1.3.3. Activating dendritic cells *ex vivo*

In order to generate *ex vivo* a population of antigen-loaded DCs that can stimulate robust and long-lasting $CD8^+$ and $CD4^+$ anti-tumour T cell responses in cancer patients upon vaccination, DCs need to be appropriately activated to become potent APCs. Increasing evidence showed that immature DCs can induce T cell tolerance and deletion, and help in the development of Tregs (Brocker, 1999; Dhodapkar *et al.*, 2001; Jonuleit *et al.*, 2000; Steinman *et al.*, 2003). Hence, mature DCs are preferred as vaccines. The most widely used maturation protocol in clinical studies for human monocyte-derived DCs consists of TNF-α, IL-1β, IL-6, and PGE_2, collectively known as the monocyte-conditioned media mimic or cytokine cocktail (Jonuleit *et al.*, 1997). However, a recent phase III clinical trial failed to show that vaccinating melanoma patients with cytokine cocktail-matured DCs provided benefit over standard dacarbazine chemotherapy (Schadendorf *et al.*, 2006), raising questions whether the use of PGE_2 would mediate T helper (Th) 2 polarisation and promote the secretion of IL-10 by DCs (Morelli and Thomson, 2003). Furthermore, another study showed that such cytokine cocktail-matured DCs were more effective than immature DCs in expanding Tregs (Banerjee *et al.*, 2006).

An alternative maturation protocol was described by Mailliard *et al* and consisted of 5 reagents: TNF-α, IL-1β, Poly I:C, IFN-α, and IFN-γ (Mailliard *et al.*, 2004). *Ex vivo* study demonstrated that DCs matured with this cytokine/stimulator cocktail were superior in stimulating CTL responses than DCs matured with cytokine cocktail containing PGE_2 and no Poly I:C. In addition, the DCs responded to CD40 stimulation and produced IL-12. TLR agonists such as poly I:C are the subject of intensive investigation since TLR ligation and subsequent signalling can lead to

potent DC maturation (Akira *et al.*, 2006; Iwasaki and Medzhitov, 2004). DCs cultured with microbial TLR ligands or their pharmacological analogs, such as the TLR4 ligand lipopolysaccharide (Kisseleva *et al.*, 2006) and monophoshoryl lipid (MPL), the TLR3 ligand Poly I:C, the TLR9 ligand oligodeoxynucleotide containing one or more unmethylated CpG dinucleotides (CpG ODN), and the TLR7/8 ligands R848 and imiquimod, undergone maturation as evidenced by upregulation of their costimulatory molecules. In one study, immature *ex vivo*-generated DCs injected into the skin of mice previously treated with imiquimod to induce a local inflammatory reaction showed that the DCs migrated to lymph and induced CTL responses (Martin-Fontecha *et al.*, 2003; Nair *et al.*, 2003). Indeed, MPL has already been approved for use in clinical trials (Neidhart *et al.*, 2004; Vantomme *et al.*, 2007). Importantly, DC maturation can be augmented even further by using combinations of TLR agonists (Gautier *et al.*, 2005; Napolitani *et al.*, 2005; Warger *et al.*, 2006).

An alternative mode of DC maturation is ligation of CD40. Signalling through CD40 has multiple effects on DCs, including inducing the upregulation of costimulatory molecules, the secretion of cytokines (notably IL-12), and the upregulation of several anti-apoptotic molecules, all of which are required by DCs to optimally activate $CD8^+$ and $CD4^+$ T cells (Grewal and Flavell, 1998; Quezada *et al.*, 2004). It has been demonstrated that DCs activated with anti-CD40 antibodies or CD40 ligand enhanced their costimulatory molecules expression and resulted in the induction of potent immune responses (French *et al.*, 2007; Rew *et al.*, 2005; Watanabe *et al.*, 2003). Hanks *et al* developed a drug-inducible CD40 expression system to specifically augment CD40 signalling of DCs after *in vivo* administration (Hanks *et al.*, 2005). It was shown that DCs expressing the modified CD40 molecules (made by fusing a

membrane-localised cytoplasmic domain of CD40 to a drug-binding domain) would selectively undergo maturation in the draining lymph node via CD40 trimerisation upon injection of the appropriate drug.

1.3.4. Overcoming tolerance

Because tumours are intrinsic, generating an effective anti-tumour response requires mounting an immune attack which may require breaking self tolerance. Although not all tumour antigens are self antigens (e.g. Epstein Barr Virus antigens in some lymphomas and mutated p53 in many epithelial carcinomas, as discussed in section 1.3.1), it has become clear that inefficient tumour rejection can result not as a result of insufficient effector cells (Gajewski *et al.*, 2006; Wick *et al.*, 1997) but because of the immunosuppressive mechanisms exerted by the tumour itself (see section 1.2) and by the cells, such as Tregs, that are present in the tumour microenvironment. $CD4^+$ $CD25^+$ Tregs have emerged as important players in suppressing host anti-tumour responses (Terabe and Berzofsky, 2004; Wang and Wang, 2007; Zou, 2006). They act through heterogeneous mechanisms such as direct contact or the production of soluble factors such as IL-10 and TGF-β to inhibit the function of T cells (Somasundaram *et al.*, 2002; Woo *et al.*, 2002; Yang *et al.*, 2006c), NK cells (Ghiringhelli *et al.*, 2005), B cells (Lim *et al.*, 2005) and DCs (Larmonier *et al.*, 2007). Numerous studies have shown that the numbers of $CD4^+$ $CD25^+$ Tregs are increased in the blood (Javia and Rosenberg, 2003; Liyanage *et al.*, 2002; Somasundaram *et al.*, 2002; Wolf *et al.*, 2003; Woo *et al.*, 2001), tumour and draining lymph nodes (Alvaro *et al.*, 2005; Lee *et al.*, 2005; Yang *et al.*, 2006a; Yang *et al.*, 2006b) of patients with different cancers.

Depleting Tregs in mouse models of cancer has been shown to be an effective anti-cancer treatment (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999). Denileukin diftitox (Ontak) is a recombinant fusion protein consisting of IL-2 and diphtheria toxin that has been approved by the Food and Drug Agency (FDA) for treating cutaneous T cell leukaemia/ lymphoma (Eklund and Kuzel, 2005; Turturro, 2007). As cutaneous T cell leukaemia/ lymphoma is characterised by a large number of malignant $CD4^+$ $CD25^+$ T cells, Ontak works by binding the IL-2 receptor chain of CD25 on these malignant $CD4^+$ $CD25^+$ T cells to inhibit their protein translation and cause them to undergo apoptosis. Due to its high affinity to the IL-2 receptor, Ontak is being used in the clinics for inhibiting and depleting Tregs in patients with renal cell carcinoma (Dannull *et al.*, 2005) and melanoma (Mahnke *et al.*, 2007) with some degree of success. To further augment the Treg depletion regimen, it is important to block VEGF (Li *et al.*, 2006a), prostaglandins (Mahic *et al.*, 2006) and estrogen (Polanczyk *et al.*, 2004) that are important for Treg differentiation and functions. In mouse models of colon cancer and melanoma, blocking VEGF reduced the number of intratumoral Tregs (Li *et al.*, 2006a). Cyclophosphamide, a standard cytotoxic chemotherapeutic, can also inhibit Tregs (Berd and Mastrangelo, 1987; Ghiringhelli *et al.*, 2007).

T cells in chronic viral infections and in the tumour setting are frequently impaired in their functions and express high level of programmed death-1 (PD1), which is an inhibitory molecule induced after T cell activation (Freeman *et al.*, 2000). PD1 interacts with its ligands B7-H1 [also known as PD-L1] and B7-DC [also known as PD-L2] (Dong *et al.*, 2002; Iwai *et al.*, 2002). In HIV infections, PD1 expression correlates positively with plasma viral load and inversely with $CD4^+$ T cell numbers

(Day *et al.*, 2006) and hence is used as a marker of disease progression. It was shown that blocking B7-H1 with specific antibodies augmented HIV-specific $CD4^+$ and $CD8^+$ T cell function *ex vivo* (Day *et al.*, 2006; Petrovas *et al.*, 2006; Trautmann *et al.*, 2006). In the tumour setting, B7-H1 is expressed by mouse melanoma cells and their *in vivo* growth is inhibited by administering B7-H1-specific antibody (Iwai *et al.*, 2002). Most human epithelial tumours and APCs express B7-H1 and B7-H4 (Kryczek *et al.*, 2006b; Kryczek *et al.*, 2006a; Tringler *et al.*, 2005) and represent attractive targets for cancer immunotherapy. PD1 blockade is currently being evaluated in humans, and it remains to be seen whether blockade of other functionally related coinhibitory molecules, such as B7-H3, B7-H4 (also known as B7x or B7S1), B and T lymphocyte attenuator (BTLA), and the recently described V-set and Ig domain-containing 4 [VSIG-4] (Vogt *et al.*, 2006) will show significant anti-tumour effects in humans.

It is unlikely that one form of immunotherapy will successfully induce the regression of bulky tumours. Hence combining vaccines (DC or otherwise) with strategies to overcome tolerance should be considered. One promising strategy is the co-administration of antagonistic antibodies specific for the inhibitory signaling molecule, cytotoxic T lymphocyte-associated 4 (CTLA-4), that is expressed on activated T cells (Melero *et al.*, 1997). In mouse models, blocking CTLA-4 antibody used in combination with a GM-CSF- transduced tumour cell vaccine reduced the number of intratumoural Treg and enhanced the rejection of poorly immunogenic tumours (Quezada *et al.*, 2006). CTLA-4 blockade also released DCs from B7-mediated engagement with either effector T cells or Tregs, which causes the induction of IDO and immune suppression due to tryptophan depletion and production of pro-

apoptotic factors (Fallarino *et al.*, 2003). In humans, objective tumour regression was observed in some melanoma patients who received both the peptide/adjuvant vaccines and antagonistic CTLA-4 antibody (Hodi *et al.*, 2003; Phan *et al.*, 2003). An ongoing phase III clinical trial will resolve whether CTLA4-specific antibodies as a single agent or administered in combination with a peptide vaccine are effective in treating metastatic melanoma.

1.4. Cancer of the Ovary

1.4.1. Immunobiology of ovarian carcinoma

Ovarian cancer is the commonest female gynaecological malignancy and the 4th most common cause of cancer death for women in Europe and the United States (Yancik, 1993). Nearly 90% of all ovarian cancers are epithelial ovarian carcinoma (EOC). As ovarian cancers are often asymptomatic in the early stage, most patients present with advanced disease at the time of diagnosis. Many patients respond well to surgery and platinum-based chemotherapy with more than 50% of them entering remission. However, the majority develops recurrent chemotherapy-resistant disease. The median survival time is approximately 24 months and the 5-year survival rates for patients with stage III and IV disease are 25% and 5%, respectively. The clinical course of ovarian cancer is often marked by periods of relapse and remission. Patients in remission with minimal disease burdens are attractive candidates for evaluating immunotherapies.

Accumulating evidence shows that ovarian tumours are recognised and attacked by the immune system. Tumour-infiltrating lymphocytes (TILs) are frequently observed in ovarian tumours and shown to express IFN-γ, IL-2 and lymphocyte-attracting

chemokines (Zhang *et al.*, 2003). Such TILs are able to recognise tumour antigens (Fisk *et al.*, 1995; Peoples *et al.*, 1995), undergo oligoclonal expansion (Halapi *et al.*, 1993) and display tumour-specific cytolytic activity *ex vivo* (Freedman *et al.*, 1994; Ioannides *et al.*, 1991a; Ioannides *et al.*, 1991b). When expanded *ex vivo*, the T cell clones are able to recognise different antigenic determinants within the same patients, indicating the heterogeneity of the response within individuals (Ioannides *et al.*, 1991b). The presence of TILs is associated with significantly longer clinical remission after chemotherapy as well as improved overall survival of the patients (Zhang *et al.*, 2003). This strongly suggests that treatment of ovarian carcinoma, as of other tumours such as melanoma, breast, prostate, renal cell, oesophageal and colorectal carcinoma (Marrogi *et al.*, 1997; Naito *et al.*, 1998; Nakano *et al.*, 2001; Schumacher *et al.*, 2001; Vesalainen *et al.*, 1994), could greatly benefit from strengthening tumour rejection through immunotherapy.

The immune response in the ovarian tumour microenvironment is modulated by interactions amongst different cell types present. Curiel *et al* showed that $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs accumulated in malignant ascites and tumour of patients with advanced stage (III and IV) ovarian cancer and were involved in tumour evasion (Curiel *et al.*, 2004). The Tregs were found in close proximity with $CD8^+$ $CD3^+$ T cells, and inhibited the latter's proliferation and production of IL-2 and IFN-γ *in vitro*. They also expressed CCR4, which is the receptor for CCL22 and CCL17 chemokines, and were recruited into the tumours via CCL22 produced by tumour-associated macrophages. Blockade of CCL22 has been shown to reduce Treg trafficking *in vivo* in NOD (non-obese diabetic)/SCID mice carrying ovarian cancer xenografts (Disis *et al.*, 2006). In a transgenic mouse model of intraperitoneal cancer, Tregs inhibited anti-

tumour immunity via CTLA-4-mediated contact inhibition of immune effector T cells (Disis *et al.*, 2006). Thus it should be beneficial to inhibit the function of intratumoural Tregs in ovarian carcinomas.

Tumour-associated macrophages are a prominent component of ovarian cancer stroma and contribute to tumour progression (Kryczek *et al.*, 2006b). They were shown to express B7-H4 to suppress the anti-tumour T cell response, and blocking B7-H4 restored the T cell stimulating capacity of these macrophages and resulted in tumour regression *in vivo*. IL-6 and IL-10, that were present in high levels in the tumour microenvironment, were found to stimulate B7-H4 expression on the macrophages. In contrast, GM-CSF and IL-4, which were limited in the microenvironment, inhibited B7-H4 expression. Hence blocking B7-H4 or depleting B7-H4 expressing tumour macrophages might enhance anti-ovarian cancer T cell responses. In addition, ascites in patients with ovarian cancer was found to contain pDCs that were capable of inducing $CD8^+$ Tregs in the tumour (Wei *et al.*, 2005). Such $CD8^+$ Tregs expressed IL-10, CCR7 and CD45RO, and significantly suppressed conventional DC-mediated anti-tumour T cell effector functions through IL-10. Hence, ovarian tumours can manipulate the function and distribution of different DC subsets in the microenvironment to subvert anti-tumour immunity. It has also been shown that malignant ovarian epithelial expressed TNF-α which in turn caused a greater release of chemokines CCL2 and CXCL12, IL-6, macrophage migration-inhibitory factor (MIF) and VEGF that were required for tumour progression and angiogenesis (Kulbe *et al.*, 2007). Therefore a more complete understanding of ovarian tumour immunobiology is essential for developing effective immunotherapeutic strategies.

1.4.2. Ovarian tumour-associated antigens

1.4.2.1. CT antigens: NY-ESO-1, LAGE-1, MAGEs and SSX

Odunsi *et al* examined NY-ESO-1 and LAGE expressions by reverse transcription-PCR (RT-PCR) and immunohistochemistry in a panel of EOC tissues, and found that 82 of 93 (88%) specimens were positive for NY-ESO-1 and 22 of 107 (21%) were positive for LAGE-1 (Odunsi *et al.*, 2003). About 30% of the patients with NY-ESO-1 or LAGE-1 expressing tumours also developed antibodies to the antigens that were present for up to 3 years after initial diagnosis (Murray *et al.*, 2002). MAGE-1 is expressed in 11 of 25 benign lesions, and 15 of 27 malignant specimens (Gillespie *et al.*, 1998). MAGE-2, -3, -6 were also expressed in 5, 11 and 4 of 58 ECO, respectively (Yamada *et al.*, 1995). MAGE-4 was present in 57% of the serous carcinomas analysed and its expression was inversely correlated to patient survival (P= 0.016) (Yakirevich *et al.*, 2003). The breakpoint genes *SSX-1*, *SSX-2*, and *SSX-4* were expressed in 2.5%, 10%, and 16% of 120 EOC specimens, respectively. Two patients had antibodies to *SSX-2* and *SSX-4*, while two novel HLA-DR restricted *SSX-4*-derived T cell epitopes were also identified (Valmori *et al.*, 2006). NY-ESO-1 peptide has been evaluated in clinical trials (Odunsi *et al.*, 2007) (see section 1.5.3.4).

1.4.2.2. CA125

CA 125, a large membrane glycoprotein (>1000 kDa) encoded by the *MUC16* gene, is expressed by epithelial cells of different organs (O'Brien, 1998). It is consistently elevated in ECO and is used as the gold standard for monitoring patients' prognosis, disease progression and response to chemotherapy. Antibody regimes targeting CA125 have been developed and assessed in ovarian cancer clinical trials (see section 1.5.3.3).

1.4.2.3. Differentiation antigen: carcinoembryonic antigen

Carcinoembryonic antigen (CEA) is a 180 kDa onco-fetal glycoprotein expressed in the normal fetal colon and adult colonic mucosa. It is over-expressed in many tumours including colon, pancreas, breast, lung and ovarian carcinomas. Approximately 67% of ovarian tumours expressed CEA (Lagendijk *et al.*, 1998; Multhaupt *et al.*, 1999). It is also detected in the sera of ovarian cancer patients and elevated level is indicative of progression of the disease (Tholander *et al.*, 1990).

1.4.2.4. Human epidermal receptor-2/ neurological (HER-2/neu)

HER-2/neu protein, also known as ErbB2 receptor, is a 185-kDa transmembrane glycoprotein with tyrosine kinase activity and extensive homology to the epidermal growth factor receptor (Bargmann *et al.*, 1986; Coussen *et al.*, 1985). It is involved in the proliferation, differentiation and survival of normal cells. It is frequently amplified and overexpressed in approximately 20% of ovarian cancers (Slamon *et al.*, 1987) and in other tumours including breast (Slamon *et al.*, 2001), pancreatic (Yamanaka *et al.*, 1993) and colorectal carcinomas (Maxwell-Armstrong *et al.*, 1998). The humanised mAb, Trastuzumab (Herceptin), has been developed and used for treating early-stage breast cancer that overexpresses HER-2/neu (Mir *et al.*, 2007; Valabrega *et al.*, 2007). Data from large phase III trials showed that its use in the adjuvant setting significantly improved the overall survival of the patients (Valabrega *et al.*, 2007). Several MHC class I and II-binding synthetic peptides derived from the HER-2/neu protein sequence (Tables 1.1 and 1.2) have been successfully tested in ovarian cancer clinical trials to induce immune responses against HER-2/neu^{+} tumours (see section 1.5.3). These peptides have also been useful for monitoring anti-ovarian tumour responses and evaluating the efficacy of ovarian cancer immunotherapies.

Table 1.1 MHC class I-restricted epitopes of HER-2/neu

HER-2/neu peptides	Peptide sequence	HLA-presenting allele	Reference
p63-71	TYLPTNASL	A24	(Shiku *et al.*, 2000)
p369-377 (E75)	KIFGSLAFL	A2, A3, A26	(Brossart *et al.*, 1998; Fisk *et al.*, 1995; Sotiriadou *et al.*, 2001)
p435-443	ILHNGAYSL	A2	(Rongcun *et al.*, 1999)
p654-662 (GP2)	IISAVVGIL	A2	(Brossart *et al.*, 1998)
p665-673	VVLGVVFGI	A2	(Rongcun *et al.*, 1999)
p689-697	RLLQETELVE	A2	(Rongcun *et al.*, 1999)
p754-762	LRENTSPK	A3, A11, A33	(Kawashima *et al.*, 1999)
p952-961	YMIMVKCWMI	A2	(Rongcun *et al.*, 1999)

Table 1.2 MHC class II-restricted epitopes of HER-2/neu

HER-2/neu peptides	Peptide sequence	HLA-presenting allele	Reference
p62-76	LTYLPTNASLSFLQD	DR4/15, DR51, DR53, DQ6/7	(Kobayashi *et al.*, 2000)
p369-386	KIFGSLAFLPESFDGDPA	DR4/5, DR51, DR53, DQ6/7	(Kobayashi *et al.*, 2000)
p688-703	RRLLQETELVEPLTPS	DR4	(Salazar *et al.*, 2003)
p776-788	GVGSPYVSRLLGI	DR7, DR51	(Sotiriadou *et al.*, 2001)
p777-789	VGSPYVSRLLGIC	DR4	(Tuttle *et al.*, 1998)
p822-836	LLNWCMQIAKGMSYL	DR1/11, DR51, DR52, DQ5/7	(Kobayashi *et al.*, 2000)
p884-899	VPIKWMALESILRRRF	DR4	(Preze *et al.*, 2002)
p971-984	ELVSEFSRMARDPQ	DR4	(Salazar *et al.*, 2003)

1.4.2.5. Tumour Mucin 1 (MUC1)

MUC1 epithelial mucin is a highly glycosylated transmembrane type I glycoprotein with a molecular weight of 250 kDa. It is expressed on the apical surfaces of most human glandular epithelial tissues (Patton *et al.*, 1995) and has a unique extracellular

domain consisting mostly of 20 to 60 tandem repeats (VTNR: variable number of tandem repeats region which is made up by a 20-amino-acid sequence of PDTRPAPGSTAPPAHGVTSA). More than 80% of ovarian tumours overexpressed MUC1 of the hypo-glycosylated form on the entire surface of the tumour cells. This occurs because of the premature termination of *O*-glycosylation of the tumour MUC1, leading to an accumulation of short carbohydrate precursors such as monosaccharides Tn (GalNAcα1-O-S/T) or disaccharide T (Galβ1-3-3GalNAcα1-O-S/T), and their sialylated forms sTn and sT, respectively (Baldus and Hanisch, 2000). This also leads to the exposure of the immunogenic PDTRP region of the tandem repeat that is hidden on normal epithelial mucins by the extensive *O*-glycosylation of peptide cores. Some cancer patients can mount a cellular and humoural response to MUC1, hence indicating its potential as target molecule for immunotherapy (see section 1.5.3). Both MHC class I and II-restricted MUC1 epitopes have been identified (Tables 1.3 and 1.4) and tested in clinical trials.

Table 1.3 MHC class I-restricted epitopes of MUC1

MUC1 peptides	Peptide sequence	Region of MUC1 where peptide is derived from	HLA-presentig allele	References
M1.1	STPPVHNV	VNTR	A2	(Brossart *et al.*, 1999)
MUC1	STPPVHGV	VNTR	A11	(Domenech *et al.*, 1995)
M1.2	LLLLTVLTV	Signal sequence	A2	(Brossart *et al.*, 1999)

Table 1.4 MHC class II-restricted epitope of MUC1

MUC-1 peptides	Peptide sequence	Region of MUC1 where peptide is derived from	HLA-presenting allele	References
MUC1	PGSTAPPAHGVT	VNTR	DR3	(Hiltbold *et al.*, 1998)

1.4.2.6. Sialyl-Tn antigen and serine proteases

Sialyl-Tn antigen (sialyla2-6Gal-NAca-Ser/Thr) is expressed in the lumen of normal secreting tissues such as salivary glands, gastric, intestinal and uterine mucosa, but is absent from most of other tissues such as normal pancreas, ovary or breast tissues (Cao *et al.*, 1996). Its expression is due to the premature sialylation of the core carbohydrate structure Gal-NAca1-O-Ser/Thr which stops further elongation of the oligosaccharide chains. It is present on the tumour MUC1 mucins of ovarian tumour cells. A synthetic STn-keyhole limpet hemocyanin (KLH) vaccine (Theratope®, Biomira) has been developed and evaluated in clinical trials involving breast and ovarian cancer patients with some degree of success (Sandmaier *et al.*, 1999). On the other hand, several serine protease antigens, e.g. hepsin, stratum corneum chymotryptic enzyme, and tumour-associated differentially expressed gene product 12 (TADG-12), TADG-14 (also called kallikrein-8 or neuropsin), TADG-15, and TADG-16 (testisin) are expressed in ovarian cancers but have very limited expression in normal ovary tissues and other normal tissues (Cannon *et al.*, 2002). They are thought to play key roles in cancer invasion and metastasis, and might present new targets for immunotherapies.

1.4.2.7. Other emerging ovarian antigens

The SEREX technology is being used to screen the sera of patients with advanced serous ovarian cancer and identified several antigens recognised only by the antibodies produced by the patients (Stone *et al.*, 2003). These antigens include p53, UBQLN1, HOXB6, TOP2A, putative helicase RUVBL, HEXIM1 and HDCMA. It is found that 40% of serous ovarian cancer patients tested had serum IgG to at least one antigen, and 14% had antibodies to two or more of these antigens. Importantly,

HOXB6, TOP2A and DDB5 are overexpressed in ovarian tumours relative to normal tissues, therefore suggesting them as potential targets for immunotherapy. In addition, some widely occurring TAAs found in other forms of tumours are also overexpressed in ovarian tumours, and are summarised in Table 1.5.

Table 1.5 MHC class I-restricted epitopes of ovarian TAAs

Gene	Peptide sequence	HLA-presenting allele	Reference
Adipophilin	SVASTITGV	A2	(Schmidt *et al.*, 2004)
AIM-2	RSDSGQQARY	A1	(Harada *et al.*, 2001)
ART-4	AFLRHAAL	A24	(Kawano *et al.*, 2000)
ART-4	DYPSLSATDI	A24	(Kawano *et al.*, 2000)
Cyp-B	KFHRVIKDF	A24	(Gomi *et al.*, 1999)
Cyp-B	DFMIQGGDF	A24	(Gomi *et al.*, 1999)
G250	HLSTAFARV	A2	(Vissers *et al.*, 1999)
Htert	ILAKFLHWL	A2	(Vonderheida *et al.*, 1999)
MUC2	LLNQLQVNL	A2	(Bohm *et al.*, 1998)

1.4.3. Immunotherapy of Ovarian Cancer

1.4.3.1. Dendritic cell-based immunotherapy

Autologous DCs pulsed with mannan-MUC1 fusion protein have been evaluated in a phase I clinical trial to treat patients with adenocarcinoma expressing MUC1. The DC vaccine elicited strong T cell IFN-γ Elispot responses and delayed-type hypersensitivity (DTH) responses at injection sites of the patients. Two patients with clearly progressive disease (ovarian and renal carcinoma) at entry were stable after the therapy (Loveland *et al.*, 2006). In another phase I clinical trial, patients with

advanced gynaecological malignancies (two patients with uterine sarcoma and six with ovarian carcinoma) were vaccinated with DC pulsed with KLH and autologous tumour antigens at 10-day or 4-week intervals. Three patients showed stable disease lasting 25 to 45 weeks, and five experienced tumour progression within the first 14 weeks. KLH-tumour lysate specific response was observed in six patients (Hernando *et al.*, 2002). The use of autologous DCs pulsed with HER-2/neu- or MUC1-derived peptides were investigated in a clinical trial involving patients with advanced breast and ovarian cancers. In 5 of 10 patients, peptide-specific CTLs were detected in the peripheral blood using both intracellular IFN-γ staining and ^{51}chromium-release CTL assays. The major CTL response *in vivo* was induced with the HER-2/neu-derived E75 and the MUC1-derived M1.2 peptide, which lasted for more than 6 months, suggesting that these peptides might be immunodominant. Antigen spreading seemed to occur as one patient vaccinated with the MUC1-derived peptides also developed CEA- and MAGE-3 peptide-specific T-cell responses after several vaccinations. Another patient immunised with HER-2/neu peptides also exhibit MUC1-specific T cell response after 7 immunisations (Brossart *et al.*, 2000).

1.4.3.2. T cell adoptive transfer

In one study, 28 patients with advanced ovarian cancer received two cycles of five daily intraperitoneal infusions of autologous *ex vivo* activated peripheral blood T lymphocytes re-targeted with bispecific monoclonal antibody OC/TR, which is directed against CD3 and the folate receptor, plus recombinant IL-2. A 27% overall response rate and 10% complete response rate which lasted 18-26 months were seen (Freedman and Platsoucas, 1996). To optimise adoptive T cell transfer strategy further, it is important to select the appropriate lymphocyte subsets for *ex vivo*

expansion, for example, TILs are preferred as they presumably contain a higher frequency of TAA-specific T cells than peripheral blood T cells. However, many $CD8^+$ TILs do not express CD28. They could be expanded by artificial APCs expressing the NKG2D ligand Letal and coated with OKT3/CD28 antibodies or even OKT3 alone (Conejo-Garcia *et al.*, 2004). In addition, Tregs could be present in high numbers in ovarian tumours (Curiel *et al.*, 2004; Woo *et al.*, 2001) and should be depleted prior to expansion of T cells for adoptive transfer. At present, several clinical trials are testing the efficacy of targeting Treg with anti-CTLA4 mAb (Hodi *et al.*, 2003; Phan *et al.*, 2003) or DAB389IL-2 diphtheria toxin fusion protein (ONTAK) directed against IL-2 receptor/ CD25 (Frankel *et al.*, 2003). Patients with recurrent, multi-drug resistant ovarian cancer have been treated in these trials. If these initial data are encouraging, suppression of Tregs will certainly be important as part of the tumour immunotherapy strategies.

1.4.3.3. Antibody therapies

Oregovomab is an IgG1k subclass murine mAb that binds with high affinity to circulating CA125 (Schultes *et al.*, 1999). 145 patients with stage III or IV EOC received intravenous oregovomab at first clinical remission (Berek *et al.*, 2004). Patients given the mAb experienced a longer period of remission (13 months) compared to those with placebo (10.3 months). Another strategy is vaccination with anti-idiotypic antibodies. The mAb Abagovomab (formally know as ACA125) that mimics a specific epitope of the tumour-associated antigen CA125 expressed by most EOC has been evaluated in clinical trials (Reinartz *et al.*, 1999). After treatment with ACA125, patients showed strong increase of intracellular IFN-γ and IL-2, and a delayed induction of T helper 2-type response that promoted antibody-mediated

immunity by B cells. In another phase I and II study of ACA125, 42 patients with advanced or recurrent EOC were given four injections of intramuscular alum-precipitated abagovomab at 2 weeks intervals (Saleh *et al.*, 1998). Anti-CA125 66.7%) and CTL responses against CA125-expressing human ovarian cancer cell lines were observed, and in patients the overall survival was also enhanced (19.9 months) compared to with placebo (5.3 months).

1.4.3.4. Peptide therapies

In a phase I clinical trial of HER-2/neu peptide vaccination in 19 patients with HER-2/neu over-expressing stage III or IV ovarian or breast cancer (Knutson *et al.*, 2001), patients received monthly vaccinations with three 15 amino acid HER-2/neu-derived peptides MHC class II 'helper' peptides, containing within each the HLA-A2 binding motifs (representing the CTL epitope) for six months. It was found that peptide-specific T cell immunity was induced 83% of the patients, and the T cell responses were long-lived and detected for more than 1 year after the final vaccination in a few patients. Another clinical trial was conducted by vaccinating patients with NY-ESO-1 peptide, $ESO_{157-170}$ which consisted of epitopes recognised by both $CD4^+$ T cells and $CD8^+$ T cells. Long-lived and $CD8^+$ and $CD4^+$ T cells specific to NY-ESO-1 were detectable in some patients up to 12 months after immunisation (Odunsi *et al.*, 2007).

1.5. Cancer of the Skin

1.5.1. Immunobiology of melanoma

The highly immunogenic cutaneous malignant melanoma is the most extensively investigated human malignancy in tumour immunology. It is well-recognised that melanoma patients might develop melanoma antigen-specific T cell responses

spontaneously in the absence of any external stimulation (Germeau *et al.*, 2005; Lonchay *et al.*, 2004). Cultured single cell suspensions derived from melanoma metastases and invaded lymph nodes for 30 to 60 days in the presence of IL-2 were often able to produce as many as 10^{10}-10^{11} T cells, with most of them exerting CTL activity on fresh tumour cell preparations (Yannelli *et al.*, 1996). Adoptive transfer of these T cells with high-dose IL-2 produced objective tumour responses in a higher proportion of the patients than with IL-2 treatment alone (Rosenberg *et al.*, 1988). The frequency of CTL precursors was higher in the TILs and in tumour-invaded lymph nodes than in PBMCs (Mazzocchi *et al.*, 1994; Romero *et al.*, 1998). Melanoma patients made tumour-free by surgery or other means mount a response to antigen(s) expressed by autologous melanoma that could be sustained for years (Sensi and Anichini, 2006). These observations prompted the exploration of immunotherapy in melanoma patients. However, the overall results from melanoma clinical trials are far from satisfactory. Like ovarian carcinoma, immunosuppressive mechanisms are present at the tumour site in melanoma and need to be overcome (Lizee *et al.*, 2006). $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in melanoma can actively subvert the actions of CD8 and CD4 anti-tumour effector T cells. Human melanoma can also produce soluble factors, such as IL-10 and TGF-β that could inhibit DC and T cell functions. They also express and secrete soluble Fas ligand, TRAIL and MICA/B that can inhibit NK and CTL functions.

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1.5.2. Melanoma-associated tumour antigens

1.5.2.1. MART-1/Melan-A

The melanoma differentiation antigen, MART-1/ Melan-A, is the most widely studied tumour antigen in melanoma. It is a type III membrane protein with a molecular mass of 22–24 kDa and is highly enriched in early melanosomes, suggesting that it might play some role in early melanogenesis (Kushimoto *et al.*, 2001). Approximately 10-75% melanoma patients have T cell responses against the antigen (Dhodapkar *et al.*, 2000; Griffioen *et al.*, 2001; Lee *et al.*, 1999; Pittet *et al.*, 1999; Scheibenbogen *et al.*, 1997). Using tetramer staining, up to 0.4% of $CD8^+$ T cells in the peripheral blood of melanoma patients were reactive to MART-1 (Pittet *et al.*, 1999). Up to 3.5% of melanoma draining lymph node $CD8^+$ T cells were MART-1 specific. Upon a short-term *ex vivo* expansion, this percentage increased up to 21% (Romero *et al.*, 1998). Further analyses showed that peripheral MART-1-specific T cells are mainly [about two-thirds] $CD28^+$ $CD45RA^{high}$ or $CD45RA^{high}$ $CCR7^+$ representing naive T cells (Hamann *et al.*, 1999; Pittet *et al.*, 1999; Pittet *et al.*, 2002; Sallusto *et al.*, 1999). However, one-third of melanA/MART-1-specific T cells are of effector memory type (Valmori *et al.*, 2002). Interestingly, about 95% of melanA/MART-1-specific T cells at the tumour site represent this effector memory subtype (Pittet *et al.*, 2002).

1.5.2.2. Tyrosinase-related proteins 1 and 2

Tyrosinase-related protein (TRP) 1 and 2 are multifunctional glycoproteins with molecular weights of approximately 60 to 70 kDa, and are essential for the biosynthesis of the skin pigment melanin (Hearing and Jiménez, 1987). These differentiation antigens are overexpressed in melanoma and are used as targets in immunotherapy of melanoma. The mouse TRP-1 and TRP-2 homologs have been

cloned and widely studied. Using intracellular cytokine and tetramer staining, Valmori *et al* demonstrated in a stage IV melanoma patient that around 5% of $CD3^+$ $CD8^+$ T cells were specific to $tyrosinase_{368-376}$ (Valmori *et al.*, 2002). Most of them were $CD45RA^+$ $CCR7^+$ granzyme B^+, that was characteristic of CTL effector cells (Campbell *et al.*, 2001; Sallusto *et al.*, 1999; Valmori *et al.*, 2002). Consistent with their phenotype, these tyrosinase-specific T cells were able to recognise and lyse tyrosinase-expressing tumour cells (Valmori *et al.*, 2002).

1.5.2.3. Melanoma antigen (MAGE)

The prototype of the cancer-germline genes is *MAGE* gene family which is composed of 24 genes that range across three subfamilies: *MAGE-A*, *-B,* and *-C*, located in three different regions of the X chromosome (Chomez *et al.*, 2001; De Plaen *et al.*, 1994; van der Bruggen *et al.*, 1991). The expression of *MAGE* genes in tumours appears to be triggered by the demethylation of their promoter, apparently as a consequence of the widespread demethylation process that occurs in many tumours, including melanoma (De Smet *et al.*, 1996). CTLs responsive against the MAGE family are very rarely found despite extensive studies (Chaux *et al.*, 1998; Dhodapkar *et al.*, 2000; Gaugler *et al.*, 1994; Griffioen *et al.*, 2001; Scheibenbogen *et al.*, 1997; Traversari *et al.*, 1992; van der Bruggen P *et al.*, 1994; Zorn and Hercend, 1999a). MAGE-A10-encoded $nonapeptide_{254-262}$ which is presented by HLA-A2.1 could be an exception. CTL responses to this peptide were detected after short-term *ex vivo* expansion in 8 of 12 patients with a MAGE-A10-expressing melanoma using tetramers of HLA-A2/ peptide $MAGE\text{-}A10_{254-262}$ complexes (Valmori *et al.*, 2001). Interestingly, samples from 3 of 10 patients whose tumors had no detectable MAGE-

A10 expression and 2 of 10 healthy donors were also positive for the MAGE-A10 tetramer (Valmori *et al.*, 2001).

1.5.2.4. NY-ESO-1

NY-ESO-1, another widely studied TAA, is often over-expressed in melanoma. Jäger *et al* demonstrated that 10 of 27 patients with tumours expressing NY-ESO-1 frequently elicited spontaneous CTL and antibody responses against NY-ESO-1 protein (Jager *et al.*, 2000a; Jager *et al.*, 2000b). Most of these NY-ESO-1-reactive T cells were $CD45RA^+$ $CD28^+$, representing a memory subset of T cells (Hamann *et al.*, 1999; Valmori *et al.*, 2000). Some melanoma patients have spontaneous responses against LAGE-1, an ORF product of NY-ESO-1 (Griffioen *et al.*, 2001).

1.5.2.5. Gp-100/ Pmel17

Gp-100 or Pmel17 is the human homologue of murine *silver*, whose disruption of function produces a silver hair color in mice (Kawakami *et al.*, 1994b). Although natural T cell responses against gp-100 is infrequent, responses against 209-217-2M, a modified peptide and gp-100_{17-25} after *ex vivo* expansion were observed in melanoma patients (Yamshchikov *et al.*, 2001).

Table 1.6 Main antigenic peptides used to vaccinate melanoma patients*

Gene	Frequency of expression in metastatic melanoma	HLA-presenting allele	Peptide sequence	Ref.
MAGE-A1	46%	A2 A3	KVLEYVIKV SLFRAVITK	(Ottaviani *et al.*, 2005; Pascolo *et al.*, 2001) (Chaux *et al.*, 1999a)

MAGE-A3	74%	A1 DP4	EVDPIGHLY KLLTQHFVQENYLEY	(Gaugler *et al.*, 1994) (Schultz *et al.*, 2000)
MAGE-A4	28%	A2	GVYDGREHTV	(Duffour *et al.*, 1999)
MAGE-A10	47%	A2	GLYDGMEHL	(Huang *et al.*, 1999)
MAGE-A12	62%	Cw7	VRIGHLYIL	(Breckpot *et al.*, 2004)
MAGE-C2	59%	A2	ALKDVEERV	(Ma *et al.*, 2004)
NY-ESO-1/LAGE-2	28%	A2	SLLMWITQC	(Chen *et al.*, 2000; Jager *et al.*, 1998; Valmori *et al.*, 2000)
Tyrosinase	>90%	A2	YMDGTMSQV	(Skipper *et al.*, 1996a; Wölfel *et al.*, 1994)
MART-1/ Melan-A	>90%	A2	EAAGIGILTV	(Schneider *et al.*, 1998)
Gp-100	>90%	A2	ITDQVPFSV	(Kawakami *et al.*, 1995)

*More information can be found at:
http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm.

1.5.3. Immunotherapy of Melanoma

1.5.3.1. Dendritic cell-based immunotherapy

To provide an option to overcome HLA restriction inherent to loading DC vaccines with peptides, Palucka *et al* vaccinated 20 patients in stage IV melanoma with autologous monocyte-derived DCs loaded with killed allogeneic Colo829 melanoma cell line (Palucka *et al.*, 2006). The antigen-loaded DCs were activated *ex vivo* with TNF and CD40 ligand. The patients had a median overall survival of 22.5 months. In 2 patients who failed previous therapy, 1 experienced complete regression and 1 partial regression lasting 18 and 23 months, respectively. 3 out of 13 patients also developed MART-1 specific T cells. One patient elicited $CD8^+$ T cell immunity specific to a novel peptide-derived from MART-1 antigen, suggesting that cross-presentation of melanoma antigens by DC vaccine had occurred. DCs loaded with

peptides derived from four melanoma antigens (MART-1, TRP, MAGE-3, and gp-100) and influenza matrix peptide (Flu-MP) have also been evaluated in the clinic (Banchereau *et al.*, 2005). 14 of 20 patients received peptide-pulsed DCs without KLH and 6 received KLH-loaded DCs. Melanoma-peptide-specific recall memory $CD8^+$ T cells secreting IFN-γ were detected in 6 of the patients. The median overall survival was 12 months and the median event-free survival was 4 months.

To determine the best route of administration of auotologous DCs pulsed with melanoma peptides (MART-1, gp-100 and TRP) *in vivo*, 27 patients with metastatic melanoma were randomly assigned to receive the DC vaccines via the intravenous [IV], intranodal [IN], or intradermal [ID] route in a phase I study (Bedrosian *et al.*, 2003). 22 of 27 (81.5%) patients completed all four vaccinations with a few patients developing some toxicity including rash, fever, and injection site reaction. All routes of administration induced comparable increases in tetramer-staining $CD8^+$ T cells (5 of 7 patients from IV route, 4 of 7 patients from IN route, and 4 of 6 patients from ID route). However, the IN route induced significantly higher number of $CD8^+$ T cells that recognised the peptide antigen and DTH response compared with other routes (6 of 7 IN patients versus 2 of 6 ID patients versus 0 of 6 IV patients; P =0.005), suggesting that IN route is the preferred route for administrating DC vaccines.

1.5.3.2. T cell adoptive transfer

In a phase I study, Rosenberg *et al* reported that 18 of 35 advanced metastatic melanoma patients treated with autologous tumour-reactive lymphocyte cultures experienced an objective clinical response [>50% reduction in tumour] (Rosenberg and Dudley, 2004). In some patients, tumour regression was accompanied by a large

in vivo expansion of the administered anti-tumour lymphocytes that persisted in the peripheral blood for many months. Another study by Morgan *et al* circumvented the requirement of expanding patients' pre-existing anti-tumour T cells *ex vivo* by culturing patients' PBMCs with IL-2 and anti-CD3, and transduced with a retroviral vector containing the gene for T cell receptor (TCR) α and β chains reactive against the melanoma antigen MART-1 (Morgan *et al.*, 2006). All 17 patients with refractory disease received fludarabine and cyclo-phosphamide as part of a lympho-depleting regimen, followed by transduced T cells and IL-2. Transduced TCRs were shown to persist in the DNA of PBMCs, and 2 of 17 patients had complete responses. This is the first published using cells transduced with specific TcR, and may lead to further advances in adoptive T cell therapy.

1.5.3.3. Peptide therapies

Meijer *et al* vaccinated stage I to III melanoma patients with the modified HLA-A2-binding gp-100(209-217-2M) peptide, and detected 3 types of responses amongst the patients (Meijer *et al.*, 2007). One group of patients responded only to the modified peptide used for immunisation, whereas another group of patients reacted to both the modified and native gp-100 peptides. In the third group of patients, circulating $CD8^+$ T cells recognised $HLA\text{-}A2^+$, gp-100 expressing melanoma cell lines and also the modified and native gp100 peptides. High avidity, tumour-reactive T cells were detected in most patients. On the other hand, most patients also had low avidity T cells that were only capable of lysing tumour cells if tumour cells were first pulsed with the native gp-100(209-217) peptides. These results indicate that vaccination with a modified gp-100 peptide induced a heterogeneous group of gp-100-specific T cells with a spectrum of functional avidities.

In a phase I clinical trial, 11 end-stage melanoma patients were vaccinated intradermally with 3 peptides: MART-1_{26-35} peptide E27L (ELAGIGILTV), tyrosinase$_{368-376}$ peptide N375Q (YMDGTMSQV), and gp-$100_{209-217}$ peptide T210M (IMQVPFSV), together with tetanus toxoid and GM-CSF (Bins *et al.*, 2007). Although all patients showed a rise in anti-tetanus IgG titres, peptide-specific $CD8^+$ T-cells were induced in only 3 patients. In 2 of the 3 patients, the response was directed against MART-1_{26-35} and made up of 0.2% and 3.3% of the $CD8^+$ population; however, in both instances these cells did not produce IFN-γ on stimulation with the unmodified peptide. The third patient mounted a small (0.1%) response against gp-100. None of the 11 patients responded clinically according to response evaluation criteria in solid tumors criteria. These results strongly suggested that peptide vaccination in itself was not potent enough as an effective melanoma immunotherapy in advanced-stage patients, and need to be combined with other forms of therapies to increase its efficacy.

1.5.3.4. Other therapies

Ipilimumab (MDX-010), a commercially available anti-CTLA-4 antibody, has been evaluated in several published phase I and phase II trials producing objective clinical responses in approximately 15% of pre-treated patients (Attia *et al.*, 2005; Hodi *et al.*, 2003; Phan *et al.*, 2003; Sanderson *et al.*, 2005). A higher response rate (22% in 36 patients) was observed when ipilimumab was used in combination with high dose IL-2 (Maker *et al.*, 2005). Ticilimumab, another anti-CTLA-4 antibody, is also being tested in a phase I trial and demonstrated equivalent efficacy to ipilimumab (Ribas *et al.*, 2005). Successful anti-CTLA-4 antibody therapies are frequently associated with side-effects such as diarrhea, colitis and autoimmune hypophysitis with symptoms

such as extreme fatigue, headaches and memory loss (Beck *et al.*, 2006; Blansfield *et al.*, 2005). While autoimmune side effects are dangerous, they are associated with clinical response and successful activation of the immune system by anti-CTLA-4. Careful monitoring of patients undergoing anti-CTLA-4 antibody treatment is essential.

IFN-α has been used in the treatment of melanoma for decades, although its application has been limited by toxicity. The immunomodulatory effects of IFN-α were demonstrated in 2 recent studies. In the first study, 20 patients with stage III melanoma were treated with high dose IFN-α for four weeks followed by lymphadenectomy. 55% of the patients had objective response rate (ORR), and 15% had a complete response. Immunohistochemical analysis also revealed T lymphocytes infiltrating the tumours (Moschos *et al.*, 2006). A second study showed that the relapse-free survival in 200 patients treated with adjuvant high-dose IFN-α correlated with the development of auto-antibodies and clinical autoimmunity (Gogas *et al.*, 2006). Together, these two studies demonstrated the promising anti-tumour activity of IFN-α in melanoma.

1.6. Hypochlorous acid (HOCl) as a link between innate and adaptive immunity

1.6.1. HOCl as potent oxidising microbicidal agent

Phagocytic cells such as neutrophils and macrophages play a key role in innate immunity in recognising, ingesting and destroying many pathogens. Upon phagocytosis, phagocytes produce a variety of reactive oxygen species (ROS), such as nitric oxide (NO), the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hyphochlorous acid (HOCl) that are directly toxic to bacteria. NO is produced by an

inducible high expression form of NO synthase (iNOS2), whilst O_2^- is generated by a multicomponent, membrane associated nicotinamide adenine dinucleotide phosphate reduced oxidase in a process known as the respiratory burst due to transient consumption of oxygen (Roos *et al.*, 2003). Then O_2^- is converted by the enzyme superoxide dismutase to H_2O_2 for killing microorganisms and for conversion by myeloperoxidase (MPO) to microbicidal HOCl (Figure 1.1).

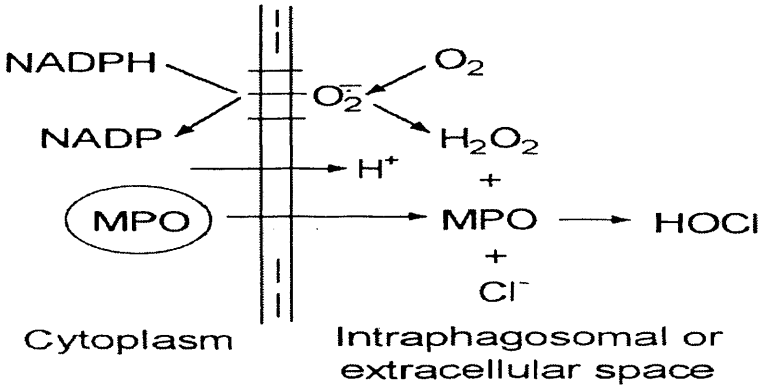


Figure 1.1 MPO-H_2O_2-chloride antimicrobial system (Klebanoff, 2005).

HOCl is the major end-product of neutrophil respiratory burst (Foote *et al.*, 1983). In the acidic phagosome, HOCl predominates and reacts with excess chloride to form molecular chlorine (Cl_2) (Hazen *et al.*, 1996b; Hazen *et al.*, 1996a; Henderson *et al.*, 1999). Both of them are short-lived but highly reactive oxidising agents that can attack microorganisms at a variety of chemical sites (Winterbourn, 2002). Numerous chemical groups on the organism, e.g. sulfhydryl groups, iron-sulfur centres, sulfur-ether groups, heme groups, unsaturated fatty acids, can be oxidised and cause the loss of microbial membrane transport (Albrich *et al.*, 1986), an interruption of the membrane electron transport chain (Rakita *et al.*, 1990), dissipation of adenylate energy reserves (Barrette, Jr. *et al.*, 1987), and suppression of microbial DNA

synthesis (Rosen *et al.*, 1990). HOCl also reacts with nitrogen-containing compounds, such as taurine which is present at a high concentration in neutrophil cytoplasm (Learn *et al.*, 1990; Schuller-Levis and Park, 2003), to form derivatives such as mono- and dichloramines that are long-lived oxidising species hence allowing the prolongation of the oxidant activity.

1.6.2. HOCl enhances the immunogenicity of protein antigens

Besides showing strong microbicidal activity, HOCl has also been shown to enhance the immunogenicity of protein antigens by several folds via oxidation *in vivo* and *ex vivo*. One possible mechanism is by tagging antigens with aldehydes. During acute inflammation, activated neutrophils produce HOCl that quantitatively deaminates serine and converting it into glycolaldehyde (Anderson et al., 1997; Anderson *et al.*, 1999; Hazen *et al.*, 1996b). One study showed that mice immunised with hen egg lysozyme (HEL) oxidised with sodium periodate ($NaIO_4$) or with neutrophil glycolaldehyde, developed enhanced T cell immune responses compared to mice immunised with unmodified HEL (Allison and Fearon, 2000). This observation was correlated with the in vitro demonstration of enhanced presentation of glycolaldehyde-modified antigen to T cells by macrophages and bone marrow-derived DCs. Oxidation by HOCl may also involve aldehyde-independent mechanisms. Proteins oxidised by HOCl were more readily taken up and processed by APC and led to enhanced activation of antigen-specific T cells in vitro (Marcinkiewicz *et al.*, 1991; Marcinkiewicz *et al.*, 1992). One reason might be that oxidation of protein antigens allow protein unfolding (e.g. by the reduction of disulphide bonds in the protein) and therefore enhances both processing and exposure of immunogenic peptides to specific T cells (Carrasco-Marín *et al.*, 1998). The heat-shock protein (Hsp) 70 might also

have a role in chaperoning polypeptides that unfolded during oxidative stress to help them regain a functional structure or by directing them to a degradation pathway (Feder and Hofmann, 1999). Myeloperoxidase activity therefore acts as a link between the innate immune response and the induction of adaptive immunity (Marcinkiewicz, 1997).

1.6.3. HOCl-oxidised proteins in pathology and breaking tolerance

ROS are involved in various physiological responses of vascular cells, such as mitosis, apoptosis, migration, hypertrophy and modifications of the extracellular matrix. They are also implicated in several major intracellular signal transduction pathways leading to changes in gene transcription and protein synthesis (Droge, 2002; Valko *et al.*, 2007). However, increasing evidence showed that ROS might play a role in disease processes where chronic inflammation is involved. Indeed, HOCl modified protein epitopes are abundantly present in inflammatory diseases such as neutrophil-mediated liver disease (Hanumegowda *et al.*, 2003), glomerulosclerosis (Malle *et al.*, 1997), and atherosclerosis (Hazell *et al.*, 1996). For atherosclerosis, numerous studies support the notion that protein and lipid components of the arterial wall undergo oxidative damage early in atherogenesis (Stocker and Keaney, Jr., 2004). The stable chlorinated product of HOCl, 3-chlorotyrosine (Hazen *et al.*, 1997; Hazen and Heinecke, 1997), is found in low-density lipids (LDLs) recovered from human atherosclerotic aorta. Its amount is 30-fold higher than that present in the LDL recovered from plasma of healthy donors, and it is also increased in the proteins of atherosclerotic verus normal aortic intima (Hazen and Heinecke, 1997).

The etiology of osteoarthritis (OA) is poorly understood, however it has been suggested that a series of inflammatory processes may be involved in initiating and propagating the disease (Benito *et al.*, 2005). MPO, HOCl and Cl_2 modified proteins (Cl-peptides) were present in the synovial fluid of 30 patients with early OA but absent in healthy individuals (Steinbeck *et al.*, 2007). This therefore suggested their pathological involvement in OA. HOCl has also been implicated in the development of rheumatoid arthritis (Hitchon and El Gabalawy, 2004). One study demonstrated that HOCl-oxidised cartilage antigen collagen type II induced the development of arthritis in rats immunised with oxidised collagen II. The immunised rats presented high IgG titre specific to oxidised and non-oxidised collagen II. Also, IFN-γ and IL-1β mRNA expressions were detected in the lymph nodes of rats 10 days after immunisation (Westman *et al.*, 2006). In addition, ROS had been identified in the synovial fluid of 90% of patients with rheumatoid arthritis (Lunec *et al.*, 1981). The ability of HOCl-oxidised antigens to break self-tolerance and induce immunity can be exploited in the context of tumour immunotherapy where induction of immunity to self antigens is often necessary.

1.7. Introduction to this Ph.D. Project

This research project has three aims. DC-based immunotherapy is a promising approach to cancer treatment and has been widely investigated in clinical trials. In order for DCs to prime a strong and durable T cell immunity against tumours, a good source of immunogens such as the allogeneic ovarian carcinoma cell line SK-OV-3 that over-expressed two important ovarian TAAs (i.e. HER-2/neu and MUC1), is needed. Live SK-OV-3 cells are poorly immunogenic, hence the first aim was to determine whether oxidation with HOCl would kill, and enhance the immunogenicity

and the uptake of these cells by DCs. Using DCs and T cells from healthy volunteers, an *ex vivo* human cell culture system was developed to study the ability of DCs pulsed with oxidised SK-OV-3 to prime autologous tumour-specific T cells. The breadth of the response was evaluated using a panel of ovarian and melanoma antigens, and compared to priming with SK-OV-3 killed with non-oxidative methods (i.e. heat or HCl). The second aim is to explore whether T cells from ovarian cancer patients in remission, that are exposed to high levels of ovarian TAAs over prolonged periods *in vivo* are tolerant or primed effectively with oxidised SK-OV-3 *ex vivo*, and reactive towards autologous tumour cells from ascites. The use of activating CD40 antibody or MPL (both approved for use in clinical trials) for maturing patients' DCs and enhancing anti-tumour T cell responses with such DCs was also investigated. The third aim was to test the efficacy of DC preloaded with oxidised B16 mouse melanoma as cancer vaccines *in vivo* in a melanoma C57BL/6 mouse model. Several parameters that could affect the overall anti-tumour response, such as the route of immunisation, the need to use DCs in the vaccination protocol, and vaccination with DCs preloaded with oxidised B16 or non-oxidised B16 (i.e. killed with heat or HCl) was assessed.

Chapter 2

Materials and Methods

Human Studies

2.1. Media

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI)-164, M199 and AIM-V media were purchased from Invitrogen, Paisley, UK. Complete DMEM, RPMI and M199 media were made by supplementing with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all obtained from Cancer Research UK, Lincoln Inn's Field, London, except FCS which was purchased from PAA laboratories GmbH, Haidmannwerg, Austria). Complete AIM-V medium was made by supplementing with 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

2.2. Cells

2.2.1. Tumour cell lines

2.2.1.1. SK-OV-3

The ovarian carcinoma SK-OV-3 (HLA-$A3^+$/$A28^+$, HER-2/neu^+, $MUC1^+$) was a kind gift from Dr. Mike O'Hare (Ludwig Institute of Cancer Research, London, UK) and were cultured in complete DMEM at 37°C, 5% CO_2.

2.2.1.2. SK-BR-3 and MDA-MB-231

The breast adenocarcinoma SK-BR-3 (HER-2/neu^+, $MUC1^+$) and MDA-MB-231 (HLA-$A2^+$, HER-2/neu^+, $MUC1^+$) cell lines were also kind gifts from Dr Mike O'Hare (Ludwig Institute of Cancer Research, London, UK). They were maintained in complete DMEM medium at 37°C, 5% CO_2.

2.2.1.3. MEL-8, MEL-11 and MEL-12

Melanoma cell lines MEL-8 (HLA-A3$^+$/A24$^+$; B7$^+$/B14$^+$, HER-2/neu$^-$, MUC1$^-$, MART-1$^+$, MAGE-1$^+$), MEL-11 (HLA-A24$^+$/A25$^+$; B18$^+$; Cw5 , HER-2/neu$^-$, MUC1, MART-1$^+$, MAGE-1$^+$) and MEL-12 (HLA-A2$^+$/A32$^+$; B14$^+$/B57$^+$, HER-2/neu$^-$, MUC1$^-$, MART-1$^+$, MAGE-1$^+$) were gifts from Dr Luciene Lopes (Department of Immunology and Molecular Pathology, UCL, London, UK) and maintained in complete DMEM at 37°C, 5% CO_2 (Palmer *et al.*, 1999).

All the cell lines above were tested regularly for *Mycoplasma* contamination and found to be negative.

2.2.2. Ovarian cancer patient ascites samples

This study was approved by the UCL Hospitals Ethics Committee (project 03/0241). Samples were collected from patients or healthy volunteers after informed consent had been obtained. Details of the patients used in the study are shown in Table 4.1. Ascites samples were collected under sterile conditions from patient 23 and 26 who had confirmed epithelial carcinoma of the ovary with peritoneal involvement. Tumour cells, collected at diagnosis and before chemotherapy, were isolated from ascites by density gradient centrifugation, washed twice in Hanks' Balanced Salt Solution (HBSS) and plated in complete M199 medium [both from Invitrogen, Paisley, UK]. The ovarian tumour cell clumps would adhere to the culture plate surface and were re-fed with fresh media after three days of culture. At least 10^8 cells from each ascites sample were frozen in 10% dimethyl sulphoxide (DMSO) in complete M199 on the day of collection for use as targets in IFN-γ ELISPOT.

2.2.3. Dendritic cell preparation and purification

Immature monocyte-derived DCs were prepared from 120 ml of heparinised fresh whole blood collected by venesection from HLA-$A2^+$ and non-HLA-$A2^+$ healthy volunteers and ovarian cancer patients after informed consent (project approved by UCL Hospitals Ethics Committee project 03/0241). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Paque (Nycomed Pharma, Oslo, Norway) density gradient centrifugation at 400 x *g* for 30 min (brake off). At least 1 x 10^7 PBMCs were cryopreserved in FCS with 10% DMSO as APCs for T cells restimulation and ELISPOT, while the rest were resuspended in complete RPMI 1640 to allow adhesion to tissue flask. After 2 h of incubation at 37°C, 5% CO_2, non-adherent cells were gently removed and frozen in FCS with 10% DMSO at -80°C as a source of T cells. The adherent cells were subsequently cultured in complete AIM-V with 100 ng/ml recombinant human GM-CSF and 50 ng/ml IL-4 [gifts from Schering-Plough Research Institute, Kenilworth, NJ. After 5 days of culture, B cells, T cells, and natural killer cells were removed by using mAb mouse anti-human CD19, CD3, and CD2, respectively, by incubating on ice for 30 min and followed by sheep anti-mouse IgG (Dynal Biotech, Oslo, Norway) at 4 x 10^6 beads/ml for 45 min at 4°C. The immature DCs were >95% pure after 7 days of culture and expressed moderate levels of HLA-DR, HLA-ABC, and CD40, low level of CD86, and no CD83, CD14 and CD19.

2.2.4. T cell isolation and purification

Cryopreserved non-adherent fraction of PBMCs was rapidly thawed, washed twice with HBSS and resuspended in complete AIM-V media. The cells were then incubated with mAb antibodies to HLA-DR, CD19 and CD14 to remove activated T

cells, B cells and monocytes, respectively. In some experiments, $CD4^+$ T cells were isolated by adding depleting anti-CD8 antibody (clone UCHT-4, Sigma-Aldrich, UK) in addition to the antibodies mentioned above. The purified whole T cell population or $CD4^+$ T cells were then added to the DC-antigen on the first day of cocultures.

2.3. Synthetic Peptides

2.3.1. HER-2/neu derived peptides

The sequences of the HLA-A*0201-restricted peptides, E75 (KIFGSLAFL) and GP2 (IISAVVGIL) (Fisk *et al.*, 1995; Peoples *et al.*, 1995), were derived from the extracellular domain of HER-2/neu. They were purchased from Alpha Diagnostic International (San Antonio, Texas, USA) with purities greater than 84% and 79%, respectively, as indicated by reverse-phase high-performance liquid chromatography (HPLC) and mass spectrometry. The MHC class II T helper peptides derived from HER-2/neu – H369 (KIFGSLAFLPESFDGDPA) and H776 (GVGSPYVSRLLGICL) (Disis *et al.*, 2002; Sotiriadou *et al.*, 2001) – were synthesised by Cancer Research UK Protein and Peptide Chemistry laboratory (Lincoln's Inn Fields, London). The H369 peptide was derived from the extracellular domain of HER-2/neu and contained a CTL epitope. It was presented by HLA-DR4, DR5, DR51, DR53, DQ6, and DQ7. The H776 peptide was derived from the intracellular domain of HER-2/neu and was presented by HLA-DR51, DR7 and DR4. Both peptides were >79% pure as determined by HPLC and mass spectrometry.

2.3.2 MUC1 derived peptides

Sequences of MUC1 derived HLA-A*0201-restricted peptides, M1.1 (STAPPVHNV) and M1.2 (LLLLTVLTV) (Brossart *et al.*, 2000; Brossart *et al.*, 2001), were

purchased from Alpha Diagnostic International. The M1.1 peptide was derived from the extracellular domain of tumour MUC1 which consisted mostly of 20 to 60 tandem repeats (abbreviated VTNR: variable number of tandem repeats region), while M1.2 was derived from the signal sequence of tumour MUC1. The purities were >79% as evaluated by reverse-phase HPLC and mass spectrometry.

2.3.3. MART-1 derived peptides

The sequence of the HLA-A*0201-restricted peptide MART-1 (EAAGIGILTV) (Romero *et al.*, 1997) was derived from melanoma, and was purchased from Alpha Diagnostic International and with a purity of >79% by reverse-phase HPLC and mass spectrometry.

All the lyophilised peptides were dissolved in at least 50% DMSO plus HBSS to a stock concentration of 1 mM, filter-sterilised with 0.2 μm filter and stored at -80°C until they were required.

2.4. Antibodies for Dendritic Cell Phenotyping

The following unconjugated mAbs were used: (a) CD1a (supernatant mouse mAb NA1/34, IgG2a) was a gift from Professor A. McMichael (John Radcliffe Hospital, Oxford, UK); (b) CD2 (mouse mAb RPA-2.10, IgG1; eBiosecience, San Diego, CA); (c) CD3 (supernatant mouse mAb UCH T1, IgG1); (d) CD14 (supernatant mouse mAb HB246, IgG2b); and (e) HLA-DR (supernatant mouse mAb L243, IgG2a) were all gifts from Professor P.C.L. Beverley (The Edward Jenner Institute for Vaccine Research, Berkshire, UK); (f) CD19 (supernatant mouse mAb BU12, IgG1); and (g) CD86 (supernatant mouse mAb BU63, IgG1) were gifts from D. Hardie (Birmingham

Medical School, Birmingham, UK); (h) CD40 (mouse mAb 5C3, IgG1; eBioscience, San Diego, CA); (i) CD83 (mouse mAb HB15e, IgG1; eBioscience, San Diego, CA); (j) HLA-ABC (mouse mAb W6/32 IgG2a; SeroTec, Kidlington, Oxford, UK); and (k) IgG2a isotype control (Dako A/S). The secondary antibody for flow cytometry was rabbit anti-mouse FITC-conjugated (Dako Cytomation, Denmark) at a 1:20 dilution of a 0.3 mg/ml stock to give a final concentration of 15 μg antibody/10^6 cells/ 100 μl.

The following PE-conjugated mAbs were used: (h) CD83 (Clone HB15e, mouse IgG1; R&D Systems, UK); (i) CD40 (Clone 82111, mouse IgG2b; R&D Systems, UK); (j) CD86 (Clone 37301, mouse IgG1; R&D Systems, UK); (k) PE-conjugated mouse anti-HLA-DR (eBioscience, San Diego, CA); (l) CD3 (Clone UCHT1, mouse IgG1; R&D Systems, UK) as an isotype control for DC phenotyping.

2.5. Western Blot Analysis of HER-2/neu and MUC1 Expression

1 x 10^6 SK-OV-3 ovarian tumour cells or 1 x 10^6 tumour cells from ovarian cancer patients' ascites were washed twice with HBSS and lysed in 20 μl of reducing sample buffer (2% sodium dodecyl sulfate [SDS; Sigma], 10% glycerol [BDH, UK], 2% β-mercaptoethanol [Sigma], 60 mM Tris–HCl [pH 6.8; Calbiochem, San Diego, CA, USA], and bromophenol blue [Sigma]). The cell lysate was sonicated for 30 sec and boiled for 8 min. It was resolved on a 7.5% SDS-polyacrylamide gel electrophoresis electrophoresis at 100V in running buffer (14.4% glycine and 3% Tris-base [both from Sigma] in dH_2O) for 1.5 h at room temperature (RT), and transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences, UK) at 30V at 4°C overnight. After protein transfer, the membrane was incubated for 1 h at RT with blocking buffer (5% skimmed milk powder [Tesco, UK] in Tris buffered saline [TBS] plus 0.1%

Tween 20 [BDH, UK]). It was then washed 4 times (5 min each with shaking) with washing buffer (TBS with 0.1% Tween 20), and incubated with relevant primary antibody solution (primary antibody in TBS containing 0.1% skimmed milk powder and 0.1% Tween 20) at 4°C overnight. HER-2/neu was detected by anti-HER-2/neu mAb (Santa Cruz Biotechnology) at a 1:100 dilution, and MUC1 was detected by HMFG2 anti-MUC1 mAb (A gift from Dr Joyce Taylor-Papadimitriou from Cancer-Research UK, GKT School of Medicine, London) at 1:10 dilution. The following day, the membrane was washed 4 times with washing buffer and incubated with horseradish peroxidase (HRP) conjugated swine anti-rabbit IgG [Dako] at 1:3000 dilution for 1 h at RT. Then membrane was washed 4 times with washing buffer, and immunodetection of the proteins was carried out by using the ECL reagent according to the manufacturer's guidelines (Amersham Pharmacia Biotech). For positive control, SK-BR-3 breast tumour cell line that highly expressed HER-2/neu and MUC1 was used. MEL-8 and MEL-11 cell lines that expressed none of the proteins were processed as described above and used as negative controls.

2.6. Immunohistochemistry of HER-2/neu and MUC1 Expression in Primary Ovarian Tumours

HER-2/neu immunostaining was carried out using the Dako Hercep Test Kit (Carpinteria, CA, USA). Paraffin sections were dewaxed, rehydrated and heated in Epitope Retrieval Solution in a water bath at 98°C for 40 min. After washing, primary polyclonal antibody was applied for 30 minutes. The staining was visualized using peroxidase coupled secondary antibodies and di-aminobenzidine. Sections were rinsed and counterstained with haematoxylin, before mounting. Controls included three breast cancer cell lines scoring 3+, 2+, 1+ and 0, and a positive and negative

tissue control section. Slides were scored based on the staining pattern. 0: No staining at all, or very slight partial membrane staining in less than 10% of tumour cells. 1+: Faint barely perceptible membrane staining in more than 10% of tumour cells or the cells are stained in only part of their membrane. 2+: Weak to moderate complete membrane staining in more than 10% of tumour cells. 3+: Strong complete membrane staining in more than 10% of tumour cells. For MUC1 immunostaining, paraffin sections were dewaxed, rehydrated and heated in retrieval solution (ERI) for 20 min at 100°C. Monoclonal MUC1 antibody (HMFG2) was used at a dilution of 1:10, for 20 min. Staining was visualized as above. Immunostaining was assessed on the basis of intensity of staining (1+ to 3+) and distribution (membrane and cytoplasmic).

2.7. Methods for Inducing Tumour Cell Death

2.7.1. Oxidative-killing with hypochlorous acid

Different concentrations of hypochlorous acid (HOCl) solutions [5 to 60 μM] were prepared by diluting the stock sodium hypochlorite (NaOCl) reagent [Sigma-Aldrich] with HBSS and added immediately to the SK-OV-3 cells to give a final cell density of 8×10^5/ml. The tumour cell suspensions were then incubated for 1 h at 37°C, 5%CO_2 with gentle agitation every 30 min to induce oxidation-dependent tumour cell death. After that, tumour cells were harvested and washed twice with HBSS for further use.

2.7.2. Killing by hydrochloric acid

To induce tumour cell necrosis, 1 M hydrochloric acid (HCl) solution was prepared by diluting the 10 M stock HCl (Sigma-Aldrich) in isotonic sodium chloride and added to SK-OV-3 cells to give a final cell density of 8×10^5/ml. The cell suspension was incubated for 1 min at RT and immediately neutralised with 1 M sodium

hydroxide (NaOH, Sigma-Aldrich). The tumour cells were washed twice with HBSS before adding to the DCs.

2.7.3. Killing by heat

To induce tumour cell death, SK-OV-3 cells were resuspended in complete AIM-V medium to a final cell density of 8 x 10^5/ml, and heated at 56°C for 30 min. Then the tumour cells were harvested and washed twice with HBSS before use.

2.8. Measuring Cell Death with Propidium Iodide Staining

Propidium iodide (PI) staining was used on ethanol permeabilised SK-OV-3 cells after HOCl-treatment to measure DNA content and to differentiate between apoptosis and necrosis. 1 x 10^6 tumour cells (pretreated with 0-100μM HOCl as outlined in section 2.7.1) were resuspended in 1 ml of HBSS, and an equal volume of 70% ice-cold ethanol was added dropwise to the cells (to a final concentration of 35% ethanol) and left on ice for 10 min to allow permeabilisation. Then, cells were washed twice with HBSS, resuspended in 250 μl of HBSS and treated with RNase (Sigma-Aldrich) [10ng/ml final concentration] and PI (Sigma-Aldrich) [1 mg/ml final concentration] for 30 min at 37°C, 5%CO_2. After that, they were harvested and analysed by flow cytometry. Apoptosis could be detected by the appearance of a sub-G_0 peak, representing partially degraded DNA. This method that measures DNA cell content directly has previously been validated by comparison to annexin V/ PI staining (Rad et al., 2003d). It was chosen in preference because oxidation might damage the annexin V binding epitope on the cell surface and hence lead to false negatives. To quantify the percentage of dead SK-OV-3 tumour cells after HOCl-treatment, 1 x 10^6 tumour cells were collected in 250 μl of HBSS and treated with RNase and PI as

described above, and analysed by flow cytometry. Dead SK-OV-3 cells were positive for PI staining while live cells, being able to exclude the PI dye, were negative.

2.9. Uptake of Tumour Cells by Dendritic Cells

To determine SK-OV-3 tumour cells uptake by DCs, tumour cells were labeled with 3' tetra-methyl-indocarbocyanine perchlorate (DiI, Sigma-Aldrich; final concentration 5 μM) in complete RPMI 1640 medium for 30 min at 37°C, 5%CO_2 before HOCl-oxidation (DiI staining of tumour cells was less efficient after HOCl treatment). After labeling and oxidation, tumour cells were collected and washed twice with HBSS before adding to the DCs at 1:1 ratio. To detect double positive DCs that had phagocytosed tumour cells, the co-cultures were incubated at 37°C, 5%CO_2 for 4 or 24 h. They were collected, blocked with staining buffer on ice for 10 min. Then cells were washed twice with staining buffer (HBSS, 10% filtered rabbit serum, 0.1% sodium azide) and incubated with mouse anti-HLA-DR for staining of DCs (30 min on ice). Cells were again washed twice to remove excess antibodies and incubated with FITC conjugated rabbit anti-mouse IgG for 30 min on ice. Parallel co-cultures were set up at 4°C for 24 h to determine the level of non-specific DiI transfer to DCs. Flow cytometry analysis was performed on the same day.

2.10. Activation of Dendritic Cell

2.10.1. Activation with oxidisied SK-OV-3

To investigate whether oxidised tumour cells were capable of inducing DC maturation, SK-OV-3 cells were treated with HOCl as described in Section 2.6.1., and cocultured with DCs at 1:1 ratio for 24 h at 37°C, 5%CO_2. After incubation, the DC-tumour cocultures were harvested and phenotyped as described in section 2.11.2.

2.10.2. Activation with CD40 agonistic antibody and monophoshoryl lipid A

DC maturation was induced using either CD40 agonistic antibodies (mouse IgG1, clone mAb89, Immunotech, Marseille, France), or a lipopolysaccharide A (Kisseleva et al., 2006a) analogue monophosphoryl lipid A (MPL: detoxified lipid A derived from *Salmonella*) [Avanti Polar Lipids, Inc., Alabaster, AL], chosen as both reagents have previously been approved for use in clinical trial (French et al., 2007b; Neidhart et al., 2004b; Tuttle et al., 1998; van Mierlo et al., 2002; Vantomme et al., 2007). DCs were cocultured with HOCl-oxidised SK-OV-3 to determine the optimal concentration of stimulus as described below, and then treated with increasing concentration of CD40 antibody (100 to 1000 ng/ml) or MPL (50 to 200 ng/ml) for 24 h at 37°C/ 5%CO_2 complete AIM-V. As a negative control, DCs were cultured in complete AIM-V. For positive control, DCs were treated with LPS (100 ng/ml) [Sigma-Aldrich] for 24 h. After activation, DCs were phenotyped as described in section 2.11.3.

2.11. Phenotypic Analysis

2.11.1. HLA-A2 typing of peripheral blood mononuclear cells

PBMCs were isolated from the whole blood of healthy volunteers and ovarian cancer patients were incubated on ice for 30 min with mAb to the HLA-A2 antigen (clone BB7.2, mouse IgG2b; Abcam PL, Cambridge, UK). The cells were then washed 2 times with cold staining buffer (HBSS, 10% filtered rabbit serum, 0.1% sodium azide) and incubated with FITC-conjugated rabbit anti-mouse IgG for a further 30 min on ice. After that, the cells were washed three times with staining buffer, fixed in 3.8% formaldehyde (Fluka) and collected the same day on a FACScan flow cytometer (Becton Dickinson). The data was analysed using the CellQuest software.

2.11.2. Phenotyping dendritic cells after exposure to oxidised SK-OV-3

To investigate whether HOCl-oxidised tumour cells were capable of inducing DC maturation, SK-OV-3 cells were treated with HOCl as described in Section 2.6.1., and cocultured with DCs at 1:1 ratio for 24 h at 37°C, 5%CO_2. After incubation, the DC-tumour co-cultures were harvested and resuspended in cold staining buffer for 15 min incubation on ice. Relevant unconjugated mAbs for DC maturation markers (i.e. CD83, CD86, CD40 and DR) were added and left on ice for further 30 min. Then two washes were done, and the cells incubated with FITC-conjugated rabbit anti-mouse IgG for 30 min on ice. After that, cells were washed twice with staining buffer and blocked with 1% normal mouse serum in HBSS (10 min on ice). PE-conjugated mouse anti-HLA-DR was added to double-stain DCs and left on ice for further 30 min. After that, the cells were washed three times, fixed in 3.8% formaldehyde and collected the same day on a FACScan flow cytometer.

2.11.3. Phenotyping dendritic cells after exposure to maturation stimuli

After treatment with CD40 agonistic antibodies or MPL, DCs were harvested and resuspended in cold staining buffer blocked for 15 min on ice, washed, and then incubated with PE-conjugated HLA-DR, CD83, CD86 and CD40 antibodies for a further 30 min on ice. The cells were washed three times in cold HBSS, fixed in 3.8% formaldehyde and collected the same day on a FACScan flow cytometer.

2.12. *Ex Vivo* Priming of Naïve T cells with Dendritic Cells Loaded with Antigens

2.12.1. Priming T cells with immature dendritic cells

A total of 2 x 10^6 autologous DCs were pulsed with 2 x 10^6 60 μM HOCl-oxidised SK-OV-3 cells overnight to allow uptake. In some experiments, 2 x 10^6 60 μM HOCl-

oxidised MEL-11 cells were used. 60 μM HOCl was chosen for tumour cell oxidation because it consistently induced >99% of necrotic SK-OV-3 tumour cell death (as determined by PI staining) and most efficiently upregulated CD40, CD83, and CD86 maturation markers on DCs. In some experiments, DCs were pulsed with either HCl-killed or heat-killed SK-OV-3, or the DCs were pulsed with HER-2/neu (E75) or MUC1 peptides (final concentration of 1 μM). After 24 h of incubation at 37°C, 5%CO_2, a total of 2 x 10^7 purified autologous T cells were added to the DC-antigen coculture and cultured in complete AIM-V medium. After 7 days, viable T cells were purified of necrotic debris by separation on Ficoll Lymphoprep and restimulated with x-ray irradiated autologous PBMCs (20 Grays) pulsed with relevant oxidised, or killed tumour cells, or peptides in AIM-V medium. Then T cells were re-purified by Ficoll Lymphoprep 4 days after restimulation and cultured in fresh AIM-V media without antigen for further 3 days. After that, viable T cells were harvested for IFN-γ ELISPOT or restimulated for another week with relevant antigens (i.e. HER-2/neu peptide [E75], MUC1 peptide [M1.2]) or media for pentamer staining.

2.12.2. Priming T cells with activated dendritic cells

2 x 10^6 autologous DCs were pulsed with 2 x 10^6 60 μM HOCl-oxidised SK-OV-3 cells or MEL-11 tumour cells for 4 hours to allow uptake. After that, activating anti-CD40 antibody (final concentration of 500ng/ml) or MPL (final concentration of 100ng/ml) was added to induce DC maturation. After 24 h, 2 x 10^7 purified autologous T cells (whole T cells or $CD4^+$ T cells) were added to the DC-antigen coculture and cultured in complete AIM-V medium. After 7 days, viable T cells were purified by separation on Ficoll Lymphoprep and restimulated with x-ray irradiated autologous PBMCs (20 Grays) pulsed with relevant oxidised tumour cells in AIM-V

medium. Four days after restimulation, T cells were purified of necrotic debris with Ficoll Lymphoprep and cultured in fresh medium with no antigen for 3 more days. Then viable T cells were either harvested for IFN-γ ELISPOT.

2.13. *Ex Vivo* Evaluation of Tumour-Specific T cell Responses

2.13.1. IFN-γ enzyme-linked immunospot (ELISPOT) assay

IFN-γ ELISPOT was performed according to the manufacturer's recommendations. The MultiScreen^{TM}-Immobilon^{TM} -P Filtration Plate (Millipore, Bedford, USA) were coated with anti-human IFN-γ capture antibody (1-D1K clone; Mabtech) at 2μg/ml in HBSS (100μl/well) overnight at 4°C. Under aseptic conditions, the plates were washed 3 times with HBSS and blocked with AIM-V medium containing 10% human AB serum for 1 h at 37°C, 5%CO_2. Then 2 washes with HBSS were done and the T responder cells were seeded in the wells at 1 x 10^5 cells/well. Cryopreserved autologous PBMCs were rapidly thawed, washed, irradiated (20 Grays) and co-cultured at 5 x 10^4 cells/well with the T responder cells in the presence of relevant antigens (i.e. graded dose of SK-OV-3 cells or MEL-11 cells oxidised with 60 μM HOCl per well, HER-2/neu, MUC1, or MART-1 peptides all used at a final concentration of 1 μM per well). To check for specificity, T responder cells in certain wells were incubated with PBMCs without antigen or in AIM-V medium only to determine the background secretion of IFN-γ. The ELISPOT plate was incubated for 40 h at 37°C, 5%CO_2. After incubation, the cells were removed by washing with MilliQ water and PBS, and the presence of IFN-γ produced by Ag-specific T cells was detected by the sequential addition of biotinylated mouse anti-human IFN-γ (2 h at RT) and followed by 4 washes with PBS (Cancer Research UK, Lincoln Inn's

Field, London), and alkaline phosphatase-conjugated streptavidin (1 h at RT) and followed by 4 washes with PBS. The number of spots corresponding to the IFN-γ producing cells was counted with an automatic plate reader (Autoimmun Diagnostica GmbH, Strassberg, Germany). Results were expressed as IFN-γ spots per 10^6 T cells.

2.13.2. HER-2/neu pentamer staining

T cells were stimulated with DCs preloaded with 60 μM HOCl-oxidised SK-OV-3 cells (1 tumour cell to 1 DC) as described in Section 2.12.1. Viable T cells were harvested and re-stimulated for a 3rd week with relevant antigens (i.e. 1 μM of HER-2/neu peptide [E75] or MUC1 peptide [M1.2], or with media) and IL-2 (5 ng/ml). At the end of 3rd week viable T cells were obtained by Ficoll Lymphoprep, and 5 x 10^5 T cells per group were washed once with staining buffer (PBS with 1% filtered FCS and 0.1% sodium azide) and stained with PE-conjugated HLA-A*0201-resticted HER-2/neu pentamer specific for KIFGSLAFL (abbreviated p-HER-2/neu $_{369-377}$) [ProImmune, Oxford, UK] for 20 min at 37°C. Cells were then counterstained with CD8-FITC (Clone LT8; ProImmune, Oxford, UK) for 30 min on ice. After washing twice with PBS containing 0.1% sodium azide, the cells were fixed with 3.8% formaldehyde and analysed by flow cytometry by appropriately gating on $CD8^+$ cells and excluding $CD4^+$ cells. T cells that were double positive for CD8 and HER-2/neu pentamer were expressed as a percentage of the total number of $CD8^+$ T cells gated.

Animal Studies

2.14. Mice

6-8 weeks old male C57BL/6 mice (H-2^b haplotype) were obtained from Charles River U.K. The mice were maintained in the Biological Service Unit in Windeyer

Institute of Medical Sciences, UCL. All the experiments were carried out under U.K. ethical guidelines.

2.15. Cells

2.15.1. Melanoma B16.F10

The mouse melanoma B16, subline F10, which overexpressed the tyrosinase-related protein 2 (TRP-2) was syngenic to C57BL/6 mice (Bloom *et al.*, 1997). It was a kind gift from Dr. Jonathan Silk (Tumour Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, U.K.) and was cultured in complete RPMI medium at 37°C, 5% CO_2.

2.15.2. Chinese hamster ovary (CHO) cells

The CHO cells were kind gifts of Dr. Yves Delneste (Centre d'Immunologie Pierre Fabre, France) and maintained in complete DMEM at 37°C, 5% CO_2.

2.15.3. Mouse bone marrow-derived dendritic cell preparation

After removing the muscle tissues from the femurs and tibias, the ends of the bones were disinfected with 70% ethanol for few seconds and rinsed twice with HBSS. One end of the bone was cut with scissor in a dish, and the bone marrow was removed using 200 µl of complete Iscove's modified DMEM (IMDM) [i.e. supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 50 µM β-mercaptoethanol] with a 1 ml syringe and 25 gauge needle. The cells were washed twice with HBSS and plated in a 6-well pate at a cell density of 5 x 10^5/ml in complete IMDM containing 20 ng/ml recombinant mouse granulocyte/ macrophage-colony stimulating factor (rGM-CSF). On day 4 of culture,

half of the media was in each well was gently removed and replenished with fresh complete IMDM containing mouse rGM-CSF. The immature DCs were >70% pure after 6 days of culture and were identified by high levels of CD11c and MHC Class II.

2.15.4. Preparation of mouse spleen cells

Fresh spleens were obtained from naïve mice (i.e. untreated mice) or mice previously vaccinated with DCs preloaded with oxidised B16 or media, or HBSS alone (i.e. no DCs). The spleen was rinsed with HBSS and cut into small pieces. They were gently mashed with a sterile 2 ml syringe plunger in a 40 μm cell strainer (BD Falcon, Bedford, MA, USA) containing 2 ml of complete RPMI to obtain single cell suspension. Then the cells were washed once with HBSS and incubated with Red Blood Cell Lysing Buffer Hybri-Max^{TM} (Sigma) for 5 min at RT. Following that, cells were washed twice with HBSS before use.

2.16. Tyrosinase-Related Protein 2 Synthetic Peptides

The MHC Class I H-2K^{b} restricted peptides derived from tyrosinase-related protein (TRP)-2 (SVYDFFVWL) (Bloom *et al.*, 1997) were synthesised by Cancer Research UK's Protein and Peptide Chemistry laboratory (Lincoln's Inn Fields, London) and were > 79% pure as indicated by reverse-phase HPLC and mass spectrometry.

2.17. Western Blot Analysis of Tyrosinase-Related Protein 2 Expression

The procedures for western blotting were exactly the same as described in Section 2.5 except with minor modifications. 1 x 10^{6} B16.F10 tumour cells were lysed in 20 μl of sample buffer, sonicated for 30 sec and boiled for 5 min. It was resolved on a 12.5% SDS-PAGE electrophoresis and transferred to Hybond-ECL nitrocellulose membrane

at 30V at 4°C overnight. The membrane was blocked overnight at 4°C and washed 4 times the following day. It was then incubated with anti-PEP8 (a kind gift from Professor Vincent J. Hearing of National Institutes of Health, Bethesda; a rabbit polyclonal antibody against the synthetic peptide PEP8 and recognised specifically TRP-2 in melanocytes (Tsukamoto *et al.*, 1992)) at 1:1000 dilution for 1 h at RT with shaking. After that, incubation with HRP conjugated swine anti-rabbit IgG [Dako] at 1:3000 dilution for 1 h at RT was done, and TRP-2 was detected by following the Amersham Biosciences enhanced chemiluminescence protocol. CHO cell line that had no TRP-2 expression were processed as described above and used as negative controls.

2.18. Activation of Mouse Bone Marrow-Derived Dendritic Cells

To determine the optimal concentrations for DC activation, day 6 mouse DCs were treated with recombinant mouse IFN-γ (100 IU/ml; Peprotech, U.K) and different concentrations of LPS (100 or 1000ng/ml; Sigma-Aldrich) or MPL (100 ng/ml, 1 or 10 μg/ml) for 24 h at 37°C, 5% CO_2. For negative control, DCs were treated with media only.

2.19. Phenotypic Analysis of Dendritic Cells

After treatment with CD40 agonistic antibodies, MPL or LPS, DCs were harvested and resuspended in cold staining buffer (HBSS, 10% rat serum, 0.1% sodium azide) and blocked for 15 min on ice. Then the cells were washed and incubated on ice for 30 min with FITC-conjugated CD80 (Clone 16-10A1, Armenian hamster IgG2), CD54 [Clone 3E2, Armenian hamster IgG1], CD86 (Clone GL1, rat IgG2a) or MHC Class II (I-A/I-E) [Clone M5/114.15.2, rat IgG2b] and double-stained with PE-

conjugated CD11c (Clone N418, Armenian hamster IgG1). All the antibodies were purchased from BD Biosciences, Pharmingen, San Diego, CA. After that, the cells were washed three times in cold HBSS containing 0.1% sodium azide, fixed in 3.8% formaldehyde and collected the same day on a FACScan flow cytometer.

2.20. *Ex Vivo* Loading of Dendritic Cells

Day 6 bone marrow-derived DCs were pulsed with 60 μM HOCl-oxidised B16.F10 at the ratio of 1:1 or with complete IMDM media for 24 h at 37°C, 5%CO_2. Then the cocultures were harvested, washed twice and resuspended in HBSS for vaccination.

2.21. Vaccination Protocols

For the intravenous route of administration, each C57BL/6 mouse was injected using a BD Micro-Fine U-100 sterile 1 ml insulin syringe (Becton Drive, NJ, USA) in the tail vein with 50 μl of HBSS containing DC/ tumour coculture (i.e. 1 x 10^6 DCs plus 1 x 10^6 oxidisd B16). For the intraperitoneal route, each mouse was injected in the peritoneum with 100 μl of HBSS containing DC/tumour coculture. For the subcutaneous route, each mouse was injected subcutaneously on the left flank with 100 μl of HBSS containing DC/tumour coculture. After 2 weeks of vaccination via the different routes, the mice were sacrificed and the spleen cells isolated for evaluation in the IFN-γ ELISPOT.

2.22. Mouse IFN-γ ELISpot

IFN-γ ELISPOT was performed according to the manufacturer's recommendations and performed as described in section 2.13.1. with minor modifications. The MultiScreen^{TM}-Immobilon^{TM}-P Filtration Plate (Millipore, Bedford, USA) were

coated with anti-mouse IFN-γ capture antibody (BD Biosciences Pharmingen, San Diego, CA) at 10 μg/ml in HBSS overnight at 4°C, and blocked with RPMI medium containing 5% FCS for 1 h at 37°C, 5%CO_2 the following day. Then 2 washes with HBSS were done and the responder cells (i.e. splenocytes from treated mice) were seeded in the wells at 1 x 10^6 cells/well. Fresh spleen cells (with red cells removed) obtained from naïve mouse were used as APCs and were cocultured with the responder cells in the presence of relevant antigens (i.e. 1 x 10^5 B16 oxidised with 60 μM HOCl per well or final of 1 μM TRP-2 peptides per well). To check for specificity, responder cells in certain wells were incubated with naïve splenocytes in the absence of antigen to determine the background secretion of IFN-γ. The ELISPOT plate was incubated for 20 h at 37°C, 5%CO_2. IFN-γ production by Ag-specific splenocytes was detected with biotin rat anti-mouse IFN-γ (BD Biosciences Pharmingen, San Diego, CA) [at 1 μg/ml] and counted as described in section 2.13.1.

2.23. Statistical Analysis

Means for different experimental groups were analysed from a minimum of three independent experiments (i.e. cells from at least three different subjects). The analysis of significance was carried out using Mann-Whitney, 1-way Anova, with Dunnett's post-hoc modification or paired Student *t*-test as detailed below.

Chapter 3

Evaluating Oxidised SK-OV-3 as Potent Immunogens

3.1. Introduction

Ovarian cancer commonly relapses after remission and new strategies to target microscopic residual diseases are required. One promising approach is to activate tumour-specific CTLs with DCs loaded with tumour cells. Immunisation with whole ovarian tumour cells that express both characterised and uncharacterised TAAs may elicit a stronger overall $CD4^+$ and $CD8^+$ immune response, and will diminish the chance of driving the emergence of escape mutations in the tumour cells. As live ovarian tumour cells are poorly immunogenic, a strategy to kill the tumour cells and at the same time enhance their immunogenicity is required. HOCl, a potent oxidant which has been shown to enhance the immunogenicity of protein antigens, is used. Hence, this study tests the hypothesis that allogeneic ovarian tumour cells, SK-OV-3, oxidised by HOCl would become potent immunogens that are efficiently taken up and cross-presented by DCs to stimulate autologous tumour-specific T cell responses.

3.2. Objectives

- To characterise and evaluate the suitability of allogeneic ovarian tumour cells, SK-OV-3, as a source of immunogens for DC-based immunotherapy of ovarian carcinoma.

- To develop an *ex vivo* human cell culture model for priming tumour-specific T cells with autologous DCs pulsed with HOCl-oxidised SK-OV-3 tumour cells, and evaluation using IFN-γ ELISpot assay and pentamer staining.

- To compare the immunogenicity of HOCl-oxidised SK-OV-3 cells to SK-OV-3 cells killed by non-oxidative methods (i.e. heat-killed and acid-killed).

3.3. Results

3.3.1. SK-OV-3 cells express HER-2/neu and MUC1 antigens

The SK-OV-3 line has been widely studied by both molecular and immunological techniques and was therefore chosen as a model source of antigen for these studies. The expression of the two epithelial tumour-associated antigens MUC1 and HER-2/neu was confirmed by immunoblotting (Fig. 3.1A, B). HER-2/neu (molecular weight approximately 185 kDa) was detected as a single band in SK-OV-3 and the breast cancer derived line SK-BR-3. MUC1 glycoproteins were detected as multiple glycosylated variants of approximately 250 kDa in both epithelial lines. Both antigens were absent from the melanoma tumour line MEL-11. SK-OV-3 has previously been shown to express HLA-A3 (zum Buschenfelde *et al.*, 2002); the absence of HLA-A2 was confirmed by flow cytometry (not shown).

3.3.2. HOCl induces cell death of SK-OV-3 cells via necrosis

The sensitivity of SK-OV-3 cells to HOCl oxidation is shown in Fig. 3.2. Cell survival and cellular DNA content were measured by PI staining of intact and permeabilised cells as described in Materials and Methods. Treatment of SK-OV-3 cells with increasing concentrations of HOCl resulted in a dose dependent increase in the % of unpermeabilised cells which took up PI (gate M1, Fig. 3.2A left column, quantified in Fig. 3.2B). Since viable cells are impermeable to PI, the % PI positive cells gave a measure of dead cells. 99% cell death or above was consistently observed at 60 μM HOCl or above, and this concentration was used in all further experiments. Extensive cell fragmentation was observed with 65 μM HOCl or higher (data not shown). Annexin V/PI staining did not show the presence of apoptotic cells in the HOCl-treated population (not shown). Lack of annexin V staining could have resulted

from oxidative damage to the annexin V binding epitope, however. The DNA content of the cells was therefore measured directly (Panel A, right column). PI staining clearly showed the presence of the G_1 and G_2 peaks, but no sub-G_0 staining corresponding to fragmented DNA from cells undergoing apoptosis [c.f. effects of DNA cross-linking agents on tumour cells, which induce apoptosis and the appearance of a clear sub-G_0 peak (Rad *et al.*, 2003)].

3.3.3. Activation of dendritic cells on exposure to oxidised SK-OV-3 cells

Previous studies from our own (Rad *et al.*, 2003) and other laboratories (Chen *et al.*, 2001; Sauter *et al.*, 2000; Slingluff, Jr. *et al.*, 2003) have suggested that necrotic cells may activate DC. DCs were therefore cocultured with HOCl-oxidised tumour cells (or LPS as a known DC activator) and the levels of cell surface CD86, CD83, and CD40 measured by flow cytometry (Table 3.1). Untreated cells had low levels of CD86, no CD83 expression, and intermediate levels of CD40. Increasing HOCl concentration significantly increased mean fluorescent intensity (MFI) for CD86 and CD40 ($P<0.05$, one-way ANOVA). There was also a small but not statistically significant ($P>0.05$) increase in the expression of CD83. However, the MFI at all concentrations of HOCl was significantly less than that induced in response to LPS ($P<0.01$, ANOVA with Dunnett's modification).

3.3.4. HOCl treatment enhances uptake of SK-OV-3 by dendritic cells

Uptake of tumour cells is a critical step in the pathway leading to both class I and class II MHC cross-priming. The ability of DC to phagocytose tumour cells was evaluated by flow cytometry, using a method developed previously (Rad *et al.*, 2003) [Fig. 3.3]. Tumour cells were identified by staining with the membrane dye DiI (FL2,

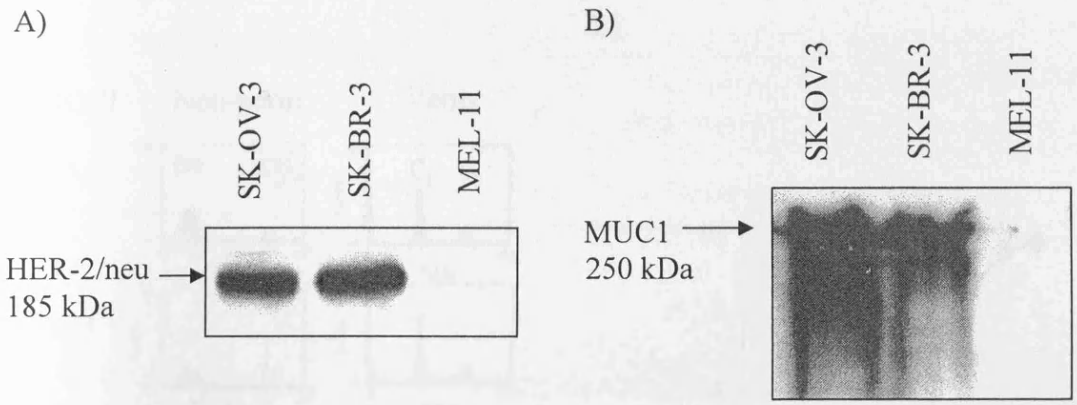


Figure 3.1 Expression of HER-2/neu and MUC1 tumour antigens on SK-OV-3 cells. A and B) 1 X 10^6 SK-OV-3, SK-BR-3, and MEL-11 cells were lysed in sample buffer, resolved on 7.5% SDS-PAGE electrophoresis, and transferred to nitrocellulose membranes. The expressions of HER-2/neu and MUC1 were detected by anti-HER-2/neu and anti-MUC1 monoclonal antibodies. Note that HER-2/neu appeared as a single band in both SK-OV-3 and SK-BR-3, while MUC1 glycoproteins were detected as multiple glycosylated variants of approximately 250 kDa in both epithelial lines. Data shown are representative of three independent experiments.

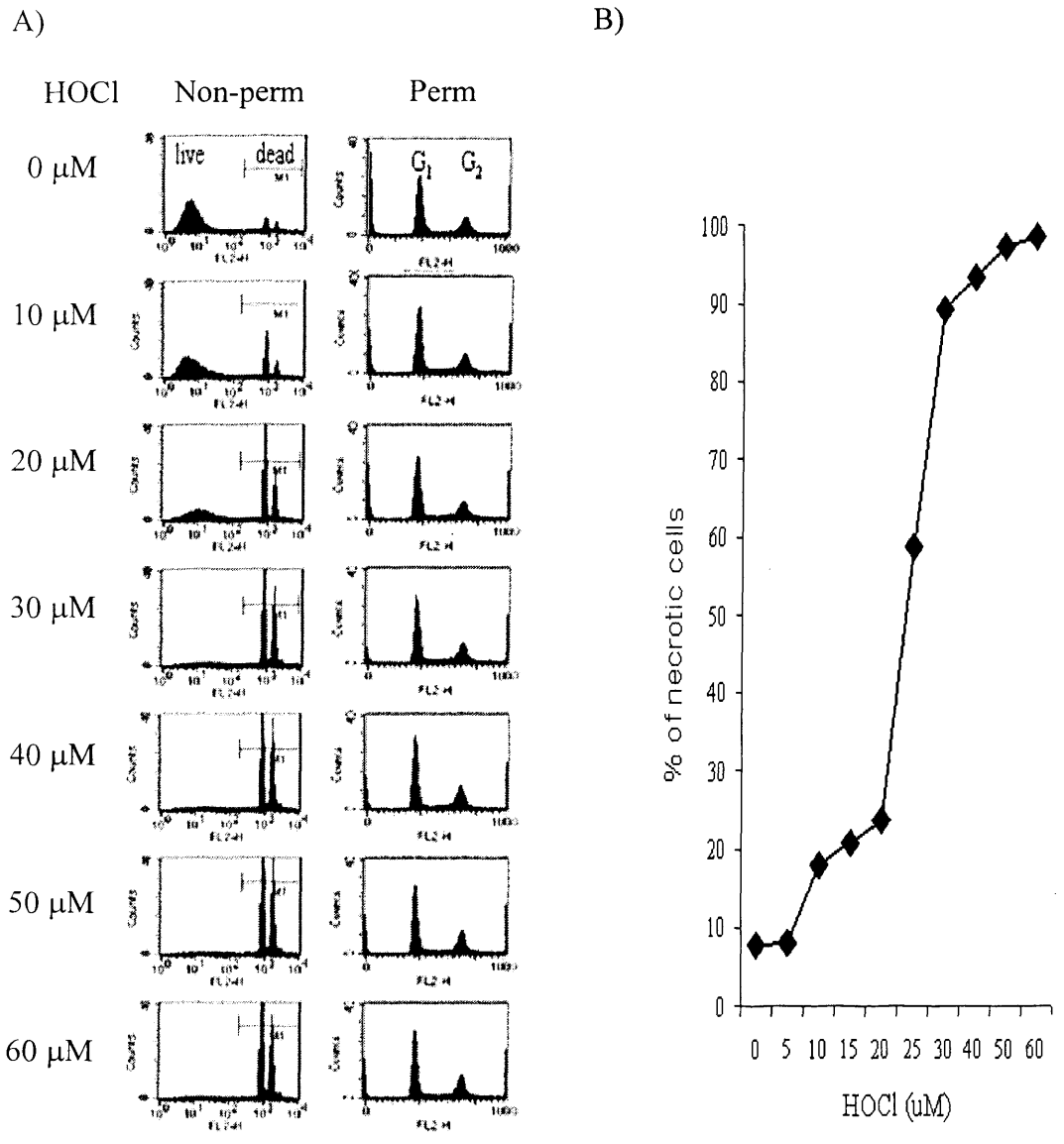


Figure 3.2 Dose dependent necrosis of SK-OV-3 cells treated with HOCl. A and B) SK-OV-3 tumour cells were incubated with different concentrations of HOCl as shown for 1 h at 37°C. Cells were washed and stained with PI as described in Materials and methods either without (non-perm, A left column) or after permeabilisation with 70% ethanol (perm, A right column). The percentage of highly PI positive cells (M1 gate) corresponded to percentage of dead cells was plotted against HOCl concentration in B. Data are representative of 5 independent experiments.

Table 3.1 HOCl-treated tumour cells induce partial dendritic cell activation

	Percentage (%) of Mean Fluorescence Intensity (MFI) of untreated DCs		
HOCl (μM)	**CD86**	**CD83**	**CD40**
0	103.4± 15.2	133.1± 29.5	100.6± 14.9
30	120.4± 20	136.1± 15.5	111.6± 11.6
40	165.5± 36	152.3± 13.8	121.2± 22.6*
50	181.9± 21.4*	160.6± 14.9	131.5± 25.3**
60	162.3± 20.5	162.3± 21.2	129.6± 11.6*
LPS matured DCs	394.0± 60.2**	355.4± 25.8**	222.6± 32.2**

SK-OV-3 tumour cells were incubated with HBSS (0 μM) or 30 μM to 60 μM HOCl for 1 h at 37°C, 5%CO_2 and cocultured with immature DCs at 1:1 ratio for 24 h. Cells were harvested and double-stained for HLA-DR and one of the markers of DC maturation CD86, CD83 or CD40. The mean fluorescent intensity (MFI) for each marker was obtained. Because the absolute MFI values showed individual variation, all values were normalised to mean MFI values of DC cultured in absence of tumour cells. Table 3.1 shows the mean ± standard error of the mean for 3 independent experiments. Asterix indicates a significant difference from DC alone, calculated using 1-way Anova with Dunnett's modification on the original unnormalised MFI values for each marker.

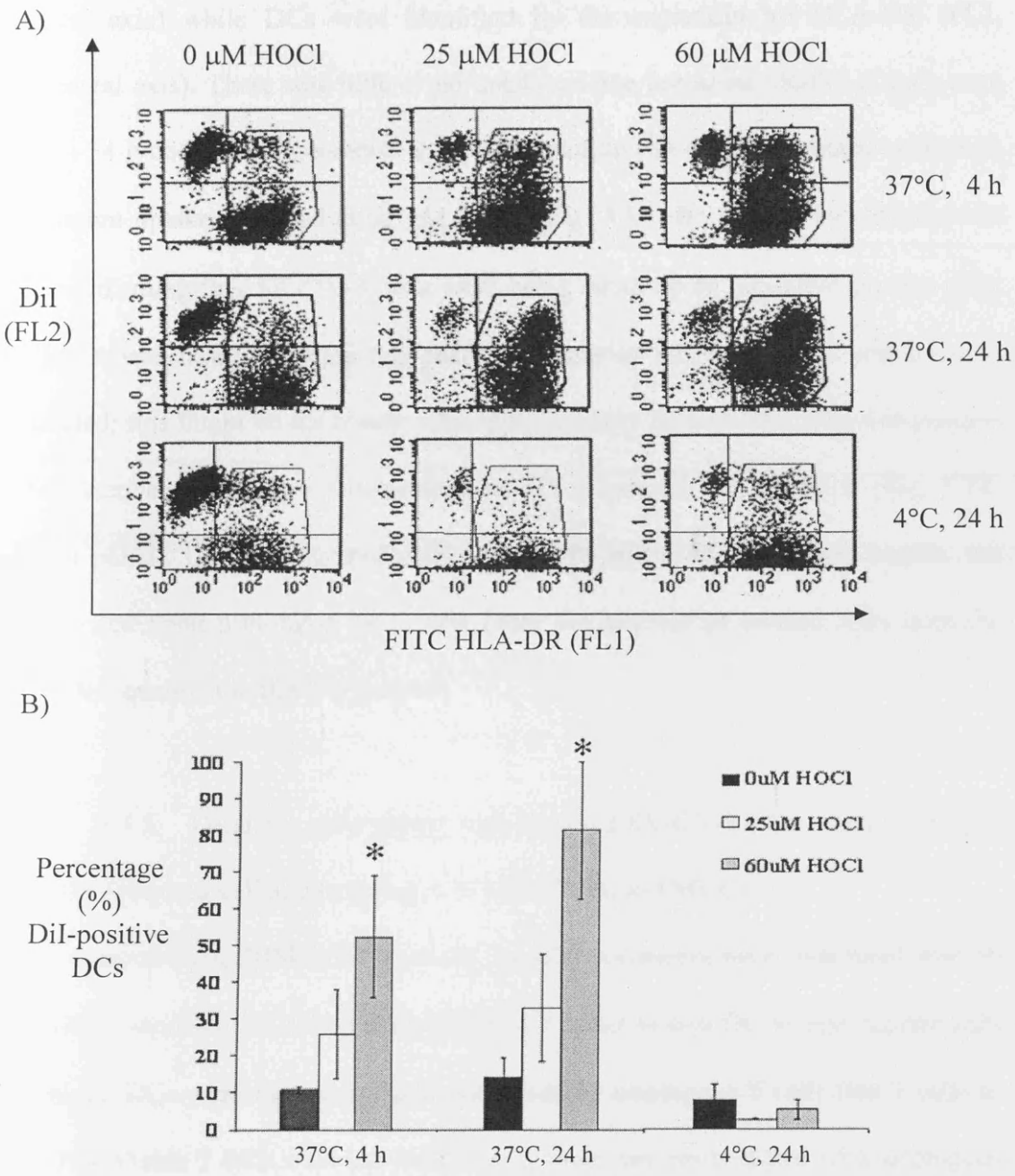


Figure 3.3 HOCl-oxidised SK-OV-3 cells are efficiently phagocytosed by dendritic cells. DiI-labeled SK-OV-3 tumour cells were treated with HBSS, 25 μM HOCl, or 60 μM HOCl for 1 h, washed and co-cultured with DCs at 1:1 ratio for 0, 4, or 24 h at 37 or 4°C. Cocultures were harvested, stained for HLA-DR, and analysed by flow cytometry. A) Representative flow cytometry profile (one of three experiments). Free residual tumor cells are shown in the *top left quadrant*, while DCs are found in the *right hand quadrants* (HLA-DR$^+$). DCs which have taken up SK-OV-3 cells are positive for both HLA-DR and DiI and appeared in the *upper right quadrant.* B) The percentage of DC (DR positive cells) which have taken up DiI under different experimental conditions was plotted against HOCl concentrations, mean ± standard error of the mean for three experiments. The asterisk shows significant difference from 0 μM HOCl within each group, Student's paired *t* test, $P<0.05$.

vertical axis) while DCs were identified by the expression of HLA-DR (FL1, horizontal axis). There was little or no uptake of live untreated SK-OV-3 cells even over a 24 h coculture. In contrast, there was rapid uptake of HOCl-treated cells, with maximum uptake observed at 60 μM HOCl (Fig. 3.3A, B). Uptake was inhibited at 4°C confirming that SK-OV-3 cells were being taken up by an active process (Fig. 3.3B). It was however noted that passive transfer of DiI to the DCs could not be excluded; this might be the reason why approximately 10% of DCs were DiI-positive when incubated with live DiI-labelled SK-OV-3 cells at 4°C for 24 h (Fig. 3.3A, lowest panel). Oxidised tumour cells, while still intact, became rather fragile, and fragmented upon prolonged incubation (note the absence of labeled cells from the upper left quadrant in the 4°C panels).

3.3.5. Dendritic cells pulsed with oxidised SK-OV-3 cells stimulate T cells specific to tumour cell and to epitopes of HER-2/neu and MUC1

DCs prepared from PBMCs of HLA-$A2^+$ healthy volunteers were cocultured with 60 μM HOCl-oxidised SK-OV-3 cells (HLA-$A2^-$, ratio of one DC to one tumour cell) overnight. DCs were then cocultured with purified autologous T cells (ten T cells to one DC). Viable T cells were harvested after 1 week and restimulated with autologous PBMCs pulsed with oxidised SK-OV-3 cells for a further 4 days, and then cultured in the absence of antigen stimulation for 3 days as described in Materials and Methods. The surviving T cells were tested by IFN-γ ELISPOT assay to assess tumour-specific T cell responses. As shown in Fig. 3.4A, T cells primed with HOCl-oxidised SK-OV-3 cells showed a significant ($P<0.05$) antigen specific recall response to the original immunogen (oxidised SK-OV-3 cells). A response was seen in all seven individuals tested, with a range of 130–220 spots/10^5 T cells.

T cells primed with oxidised SK-OV-3 gave significant IFN-γ responses to live SK-OV-3 cells in the presence of exogenous PBMC (Fig. 3.4A, second bar). This result indicates that the T cells stimulated by the oxidised SK-OV-3 cells also recognise non-modified antigens, an essential requirement if the cells are to have useful anti-tumour activity *in vivo*. In contrast, the T cells stimulated by the oxidised SK-OV-3 cells failed to respond to oxidised melanoma cells, indicating that the response showed cell type specificity.

The T cells stimulated with the oxidised SK-OV-3 cells also responded to peptides encoding known HLA-A2 epitopes of HER-2/neu and MUC1. Since SKOV-3 cells are HLA-A2$^-$, this response demonstrates that the oxidised SK-OV-3 cells had been taken up and cross-presented by the stimulating DC. The response to E75 HER-2/neu peptide was consistently better than the response to GP2 HER-2/neu peptide (three of three individuals, although the difference in the means did not reach significance; P=0.06), suggesting that a predetermined epitope hierarchy exists within the HER-2/neu antigen. The T cells did not respond significantly to an HLA-A2 melanoma peptide, confirming antigen specificity of the observed response.

T cells primed with DC in the absence of tumour cells failed to show significant recall responses to any of the antigens tested (Fig. 3.4B). Furthermore, T cells primed with live SK-OV-3 cells (X-ray irradiated in order to prevent proliferation) gave only very weak responses (less than 60 spots/10^5 cells; data not shown). It was important, to however, to determine whether the enhanced immune response seen was due specifically to oxidation by HOCl, or was simply a result of cell necrosis. SK-OV-3 necrosis was therefore induced by two other means, heat treatment (56°C, 30 min) or

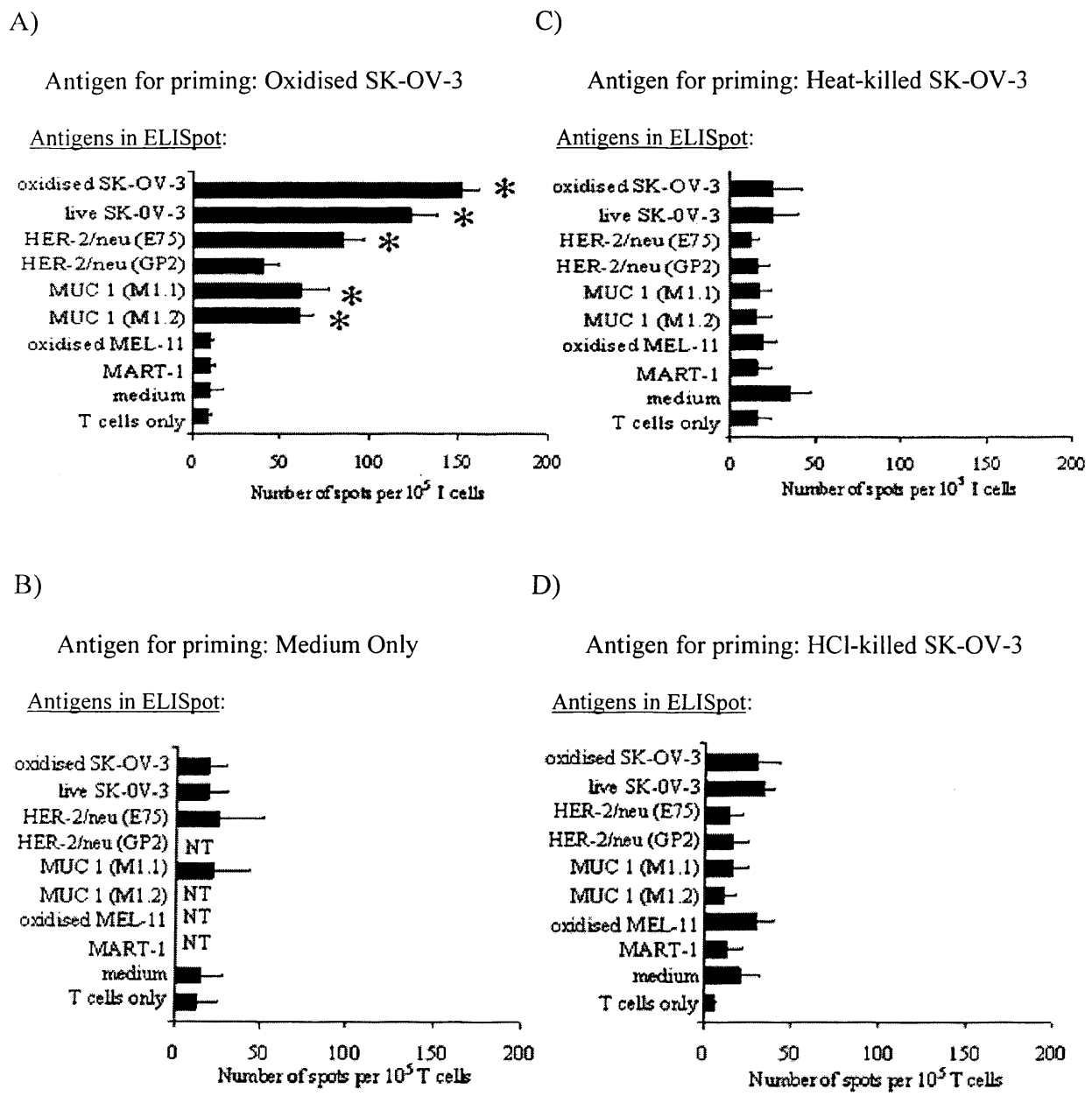


Figure 3.4 Dendritic cells loaded with HOCl-oxidised SK-OV-3 cells, but not heat-killed or HCl-killed SK-OV-3, stimulate T cell responses to tumour cells and to specific tumour antigen epitopes HER-2/neu and MUC1. T cells from HLA-A2$^+$ individuals were stimulated with autologous DCs pulsed with: A) 60 μM HOCl-treated SK-OV-3, B) complete AIM-V media without antigen, C) Heat-killed SK-OV-3 cells, or D) HCl-killed SK-OV-3 cells as described in Materials and methods. Viable T cells were harvested after 1 week and re-stimulated with autologous PBMCs pulsed with the same antigen for another 4 days, and cultured in fresh medium without antigen for three more days. IFN-γ production was measured by ELISPOT. The results are the means ± standard error of the mean for at least three independent experiments (i.e. PBMCs from different individuals). The asterisk indicates those columns differing significantly (P<0.05) from the medium only control (one-way ANOVA with Dunnett's modification for multiple comparisons).

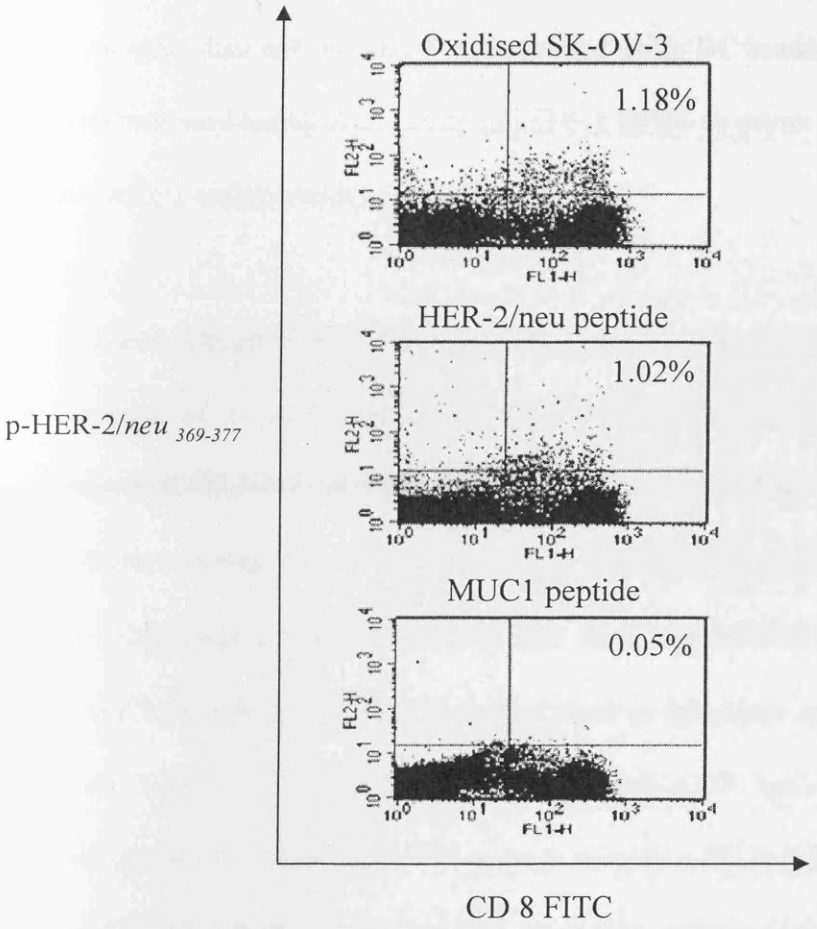


Figure 3.5 Dendritic cells preloaded with HOCl-oxidised SK-VO-3 cells cross-primed autologous $CD8^+$ T cells that were specific to HER-2/neu. T cells were co-cultured with autologous DCs (ten T cells to one DC) preloaded with HOCl-oxidised SKOV-3 cells, HER-2/neu E75 peptide or MUC1 M1.2 peptide as shown. After two further rounds of antigen stimulation (see details in Materials and methods) viable T cells were harvested and double-stained with anti-CD8 FITC and PE-conjugated HER-2/neu pentamer specific for HER-2/neu E75/HLA-A2. The percentage of total $CD8^+$ T cells positive for pentamer is shown on the top right hand corner. Data are one representative out of three independent experiments.

brief exposure to acid (1 M HCl, 60 sec). In these conditions, SK-OV-3 tumour cells retained cellular integrity but were >99% dead by necrosis (confirmed by trypan blue and PI staining, data not shown). T cells primed using DC loaded with either heat-kill (Fig. 3.4C) or acid killed (Fig. 3.4D) SK-OV-3 failed to prime a significant immune response to any antigen tested.

3.3.6. Direct versus indirect presentation of oxidised SK-OV-3 cells

The presence of HLA-A2 restricted HER-2/neu specific cells, suggesting cross-presentation of HLA-$A2^-$ SK-OV-3 cells by HLA-$A2^+$ DC, was confirmed by HLA-A2 pentamer staining. T cells were stimulated with autologous DCs pulsed with either 60 μM HOCl-oxidised SK-OV-3, HLA-A2 restricted HER-2/neu E75 peptide, or MUC1 M1.2 peptide, and expanded as described in Materials and methods. Viable T cells were harvested and double-stained with anti-CD8 antibody and HER-2/neu pentamer specific for HER2/neu E75 peptide bound to HLA-A2. T cells primed with oxidised SK-OV-3 cells, as well as HER-2/neu E75 peptide contained a population of pentamer binding CD8 positive T cells (Fig. 3.5A, top two panels) while cells primed with MUC1 peptide showed background staining (Fig. 3.5A, bottom panel). Three individuals were tested in this way, with average CD8/pentamer positive percentages of 1.42% (primed with oxidised SK-OV-3 cells), 0.77% (primed with HER-2/neu E75) and 0.02% (primed with MUC1 M1.2).

In order to determine whether oxidised SK-OV-3 cells stimulated IFN-γ release by primed T cells directly (i.e. allogeneic direct interaction), or whether T cell stimulation depended exclusively on reprocessing by autologous antigen presenting cells, T cells were cultured with HOCl-oxidised SK-OV-3 cells in the presence or

absence of HLA-A2$^+$ PBMCs as source of antigen presenting cells (Fig. 3.6). The response to HOCl-treated cells was not significantly above background in the absence of PBMCs. HOCl treatment therefore seems not only to enhance cross-presentation, but to inhibit direct allogeneic recognition. It was to be pointed out that one important control which was lacking was the use of naïve T cells or PBMCs prior to antigen stimulation to determine the precursor frequency of ovarian-specific T cells. This would be useful to determine the effectiveness of T cell priming with oxidised SK-OV-3 and to show any increase in the number of ovarian-specific T cell precursors following antigen stimulation. In Fig. 3.7, T cells from an HLA-A2$^+$ individual primed to the oxidised SK-OV-3 cells, responded directly (i.e. in the absence of exogenous PBMC) to live cells of an HLA-A2$^+$ and MUC1 and HER-2/neu expressing breast cancer cell line MDA-231 (an HLA-A2$^+$ ovarian line was not available to us) but not to an HLA-A2$^+$ melanoma cell line MEL-12.

3.3.7. Responses to oxidised tumour cells are cell type specific

The role of HOCl in enhancing tumour-specific T cell responses was tested further using an HLA-A2$^-$ melanoma cell line, MEL-11, which expresses the melanoma associated antigen MART-1 (not shown) but neither HER-2/neu nor MUC1 (Fig. 3.1). T cells primed with DC loaded with HOCl-oxidised MEL-11 cells showed strong responses to MEL-11 cells, as well as to an HLA-A2 restricted peptide derived from MART-1 (Fig. 3.8). Thus, HOCl-oxidised melanoma cells, like ovarian tumour cells, effectively stimulate antigen specific T cells via cross-priming. MEL-11 stimulated cells, however, failed to respond to oxidised SK-OV-3 cells, or any of the HER-2/neu or MUC1 peptides.

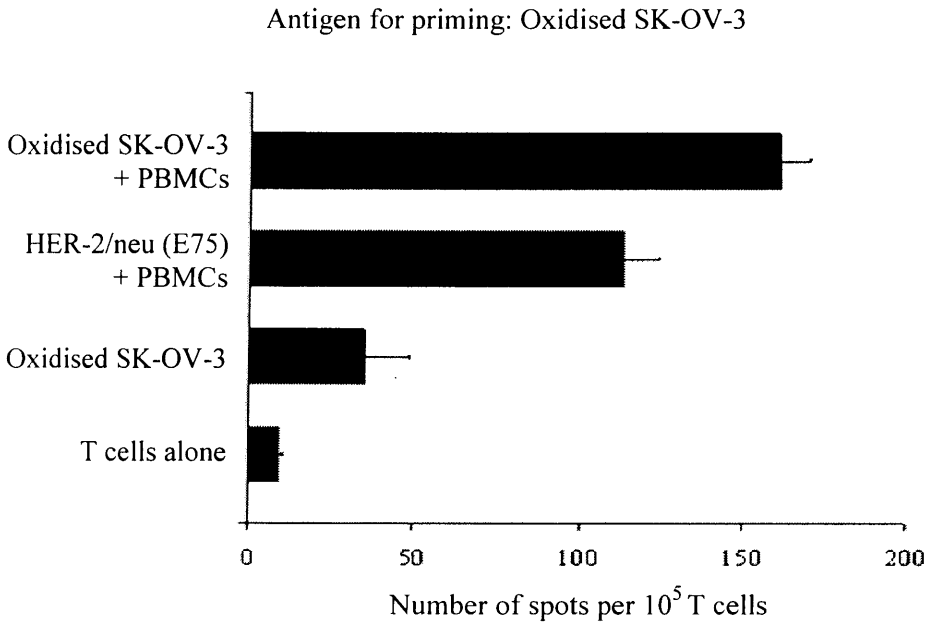


Figure 3.6 Cross-priming is necessary for presentation of HOCl-oxidised SK-OV-3 cells. T cells were primed with HOCl-oxidised SK-OV-3 cells as in Figure 3.4A and their IFN-γ responses to HOCl-oxidised SK-OV-3 or HER-2/neu peptide, in the presence or absence of autologous PBMC was evaluated with ELISPOT. The results are the means ± standard error of the mean for at least three independent experiments (i.e., PBMCs from different individuals). The asterisk indicates those columns differing significantly ($P<0.05$) from the T cell only control (one-way ANOVA with Dunnett's modification for multiple comparisons).

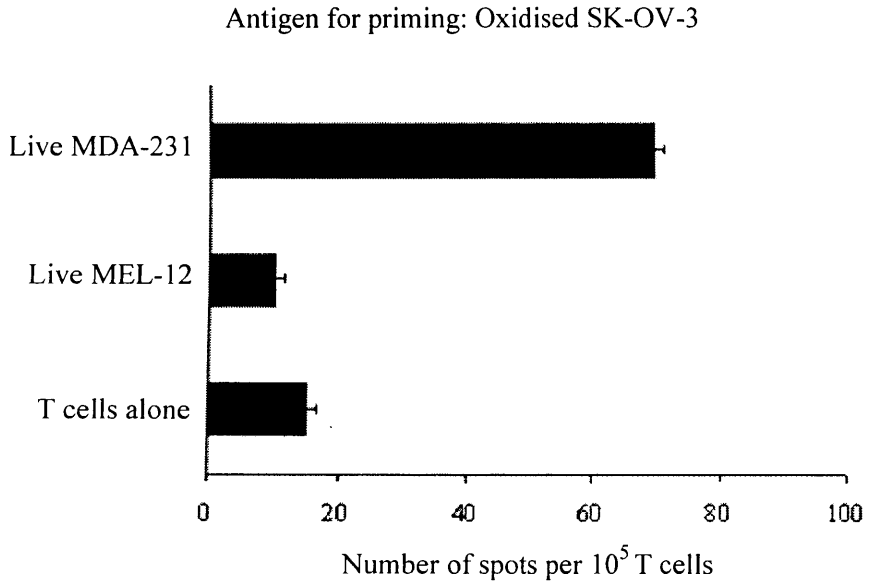


Figure 3.7 T cells stimulated with dendritic cells pulsed with oxidised SK-OV-3 cells were capable of directly recognising live HLA-$A2^+$ cell line that overexpressed HER-2/neu and MUC1. T cells were primed with HOCl-oxidised SK-OV-3 cells as in Figure 3.4A and their IFN-γ responses to live MDA-231 (HLA-$A2^+$, $MUC1^+$, HER-$2/neu^+$) or live MEL-12 (HLA-$A2^+$, $MUC1^-$, HER-$2/neu^-$) in the absence of autologous PBMC was evaluated with ELISPOT. The results are one representative experiment of two.

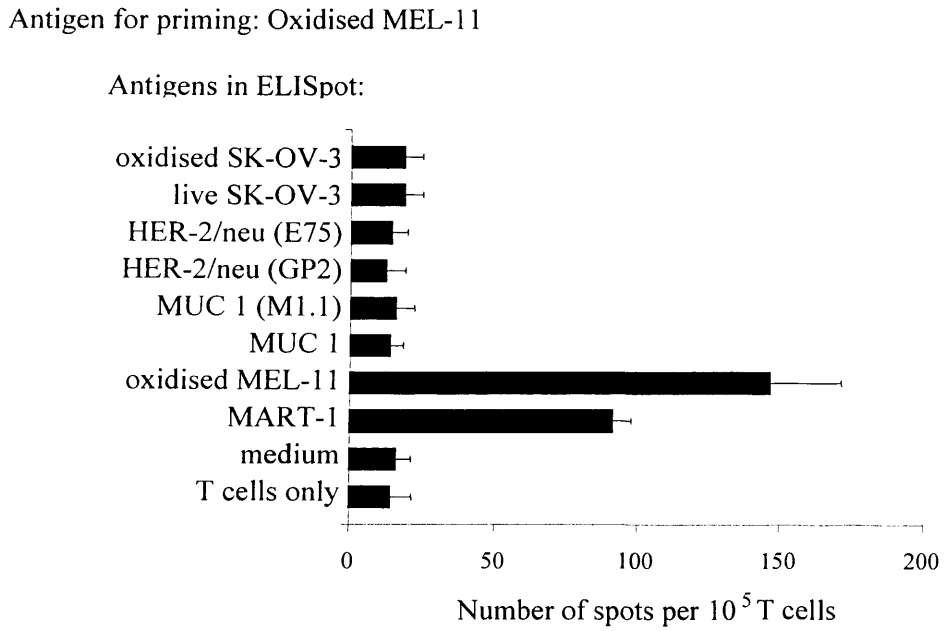


Figure 3.8 HOCl-oxidised MEL-11 cells induce melanoma specific T cells which do not cross-react with SK-OV-3 cells. T cells were primed to oxidised MEL-11 cells as described in Figure 3.4. Their IFN-γ production in response to HOCl-oxidised SK-OV-3 or MEL-11 cells, HER-2/neu peptide, MUC1 peptides, or MART-1 peptides in the presence of autologous PBMC was measured by ELISPOT. The results are the means ± standard error of the mean for at least three independent experiments (i.e., PBMCs from different individuals). The asterisk indicates those columns differing significantly ($P<0.05$) from the medium only control (one-way ANOVA with Dunnett's modification for multiple comparisons).

3.4. Discussion

Dendritic cells are key regulators of the immune system and are capable of initiating and inducing tumour-specific cytotoxic and helper T cells. Indeed, DC-based immunotherapy has been vigorously pursued in clinical trials with some success (see Chapter 1). In the case of ovarian carcinoma, DCs have been pulsed with HLA-A2 restricted peptides coding for immunodominant epitopes of either HER-2/neu or MUC1 proteins (Santin *et al.*, 2004). Though the synthesis of large quantities of clinical grade peptides is technically straightforward, there are several limitations of peptide immunogens, including the need for patients with specific HLA haplotype, limited anti-tumour responses, selection of escape variants, and lack of long-lasting memory. An alternative is to use whole ovarian tumour cells that highly express HER-2/neu and MUC1, together with many as yet undefined TAAs (Hernando *et al.*, 2002). In this study, we developed a robust *ex vivo* human cell culture system of loading HLA-$A2^+$ DCs from healthy volunteers with whole allogeneic SK-OV-3 tumour cells, and assessing their potential for generating autologous tumour-specific T cells.

The first key element of this model was that the immunogenicity of the tumour cells was enhanced by treatment with HOCl, a strong oxidising agent, which induced rapid necrosis of SK-OV-3 tumour cells. Enhancement of anti-tumour immunity thus extends previous observations of enhanced immune responses to protein antigens treated with HOCl (Allison and Fearon, 2000; Marcinkiewicz *et al.*, 1991; Marcinkiewicz *et al.*, 1992). Several studies have suggested that cell necrosis, per se, may enhance immunogenicity, perhaps by exposing heat shock proteins (Basu *et al.*, 2000; Kotera *et al.*, 2001; Sauter *et al.*, 2000). Importantly, therefore, enhanced imunogenicity to oxidised cells was seen when compared to response to SK-OV-3

cells killed by other means, such as heat or acid treatment. Since HOCl, and the closely related chemical HCl, differ principally in that the former is a strong oxidising agent, while the latter is not, these results suggest that oxidation is an essential feature of the enhancement in immunogenicity observed. Preliminary studies have shown that other forms of oxidation (e.g. by hydrogen peroxide) can also improve immunogenicity, and the precise nature of the chemical reactions responsible are being investigated further.

The mechanism for enhanced immunogenicity may be explained, at least in part, by the fact that the oxidised cells were taken up much more efficiently than live cells by DC, and experiments are underway to try and identify the receptor involved in DC uptake. However, HCl-killed or heat-killed tumour cells also showed some enhanced uptake in comparison with live cells (not shown), suggesting that other mechanisms, such as more efficient processing, may also play a role. In addition, oxidised SK-OV-3 tumour cells induced a partial activation of DCs by upregulating the maturation associated markers CD86 and CD40. DC maturation induced by oxidised SK-OV-3 cells was sub-optimal compared to the Toll-like receptor (TLR) agonist LPS, however, and additional stimulation of DC (e.g. via CD40 ligation) may further enhance the response.

A second key aspect of this model was the role of cross-presentation versus direct T cell/tumour cell interaction. The detection of responses to well-defined HLA-A2 restricted epitopes of HER-2/neu and MUC1 clearly demonstrated that oxidised SK-OV-3 cells (which were HLA-A2$^-$ and allogeneic to the DC and T cells) were efficiently cross-presented by the DC. The strong peptide-specific responses

demonstrated that a major part of the T cell response was to ''native'' cellular antigens, rather than to new neo-epitopes formed by chemical modification with HOCl. The ability of T cells primed to oxidised tumour cells to recognise live tumour targets was confirmed by using an HLA-$A2^+$ expressing breast tumour line, MDA-231, which shared both MUC1 and HER-2/neu antigen expression with SK-OV-3 (Fig. 3.7). Shared antigen expression was essential for recognition, since an HLA-A2 matched melanoma tumour was not recognised.

HOCl oxidation not only promoted cross-presentation, but also seemed to block direct allo-recognition, since the T cell IFN-γ response to oxidised cells (Fig. 3.6) showed an absolute requirement for antigen presenting cells. Unexpectedly, the T cells primed to oxidised SKOV-3 also recognised live SK-OV-3 (Fig. 3.7), even though these cells were taken up very poorly, and did not express HLA-A2. Direct recognition of SK-OV-3 cells could have occurred, however, either through a shared unknown HLA allele between volunteer and SKOV-3 (since the volunteers were typed only for the presence of HLA-A2), or via the presence of promiscuous tumour derived peptides able to interact with many different HLA alleles. The dominance of cross-presentation in this model may have important practical consequences, since it may allow the use of generic tumour-derived cell lines as immunogens, in place of patient-derived tumour cells which are more difficult to obtain and inherently more heterogeneous. The HLA of the cell line used as immunogen will in fact be irrelevant, since T cells will be primed to antigens presented in the context of the host MHC on host DCs. Such T cells will therefore be syngeneic to the primary host tumor, and therefore should be able to recognise the primary tumor directly. In this scenario a cell line would simply need to share tumour antigens with the primary tumour (e.g. MUC1 or

HER-2/neu positive tumours for SK-OV-3 priming). The use of such ''standardised'' cell lines would have major benefits in terms of practical application, since it obviates the need for patient specific tumor tissue collection and preparation. Studies to test this hypothesis, using primary autologous patient-derived tumour cells as targets following *in vitro* priming with SK-OV-3 cells are in progress.

The third important observation in this study was the tumour specificity of the responses. A persistent concern in the development of tumour immunotherapy is that the immune system will recognise and kill cells other than the tumour and hence cause autoimmune disease. This problem is particularly acute with the use of whole cell immunogens, since all cells share an enormous number of ''common'' proteins involved in housekeeping cellular metabolic functions, as well as expressing smaller numbers of ''tissue specific'' proteins. It is, therefore, of importance that in this model the response showed specificity in relation to at least two quite distinct tumour cell types. SK-OV-3 specific T cells did not respond to the melanoma line MEL-11, while conversely, T cells immunised to MEL-11 melanoma cells did not respond to SK-OV-3 cells. Further studies are in progress to identify the breadth of the response to SK-OV-3 cells, in terms of tissue specificity, tumour specificity, and its ability to recognise ''normal'' tissue.

The ability of innate immunity to drive enhanced adaptive immunity is now a central tenet of immunology. In this study, we seek to manipulate tumour immunity using HOCl, a known microbicidal product of myeloperoxidase activity in neutrophils. Although myeloperoxidase is well-established as playing a role in the effector microbicidal function of neutrophils, its role in linking innate and adaptive immunity

has only been recognised more recently (Marcinkiewicz, 1997). As discussed above, *in vivo* vaccination of patients with DCs preloaded with oxidised SK-OV-3 may induce therapeutic tumour-specific T cells responses to autologous tumor cells. Cytolysis of tumour cells by tumour-specific T cells will release further tumour antigen, and also release cytokines that drive localised inflammation. A virtuous cycle of further rounds of oxidation and immunological stimulation is thus established.

3.5. Conclusions

- HOCl in HBSS induced rapid primary necrosis of SK-OV-3 cells in a dose-dependent manner. Oxidised, but not live, SK-OV-3 cells were rapidly taken up by monocyte-derived DCs and induced partial DC maturation.

- DCs cultured from HLA-$A2^+$ healthy volunteers pulsed with oxidised SK-OV-3 (HLA-$A2^-$) stimulated autologous T cells that produced IFN-γ in response to the immunising cellular antigen and to peptides coding for MUC1 and HER-2/neu HLA-A2 restricted epitopes, demonstrating efficient cross-presentation of cell-associated antigens. In contrast, no response was seen after priming with heat-killed or HCl-killed SK-OV-3, indicating that HOCl oxidation and not cell death or necrosis per se enhanced the immunogenicity of SK-OV-3.

- Finally, T cells stimulated with oxidised SK-OV-3 showed no cross-reaction to oxidised melanoma cells, nor vice versa, demonstrating that the response was tumour-type specific.

- Therefore, immunisation with oxidised ovarian tumour cell lines may represent an improved therapeutic strategy to stimulate a polyclonal anti-tumour cellular immune response and hence extend remission in ovarian cancer.

Chapter 4

Oxidised SK-OV-3 as Cancer Vaccine to Break Tolerance in Ovarian Cancer Patients

4.1. Introduction

In chapter 3, it was demonstrated that allogeneic SK-OV-3 ovarian tumour cells oxidised by HOCl were potent immunogens for DCs in priming autologous tumour-specific T cell responses. As the studies were done using the peripheral blood of healthy volunteers, this model was tested further in the ovarian cancer setting. Ovarian cancer patients in remission are attractive candidates for immunotherapy as they have minimal tumour burden and their immune system appears to have returned to normal (Colombo *et al.*, 2006). In addition, autologous tumour cells can be obtained frequently direct from ascitic fluid as a single cell suspension, thus minimising the need for tissue disruption and manipulation, and facilitating *in vitro* restimulation assays. Furthermore, studies of the immune microenvironment in ovarian tumours showed convincing evidence for a "natural" immunoprotective response, which may be capable of enhancement by appropriate vaccination strategies (Zhang *et al.*, 2003). In this chapter, two critical questions were being addressed. First, whether T cells from patients that are exposed to high levels of ovarian cancer antigens over prolonged periods and yet are apparently tolerant to them, still respond effectively to the oxidised antigens. Second, whether the T cells stimulated in response to SK-OV-3 cross-priming actually respond to autologous tumour, which is an obvious requirement for any potential immunotherapeutic approach. It is being proposed that these studies suggest a generic method to stimulate an anti-tumour T cell response which will be capable of targeting autologous tumour in patients with ovarian cancer.

4.2. Objectives

- Using IFN-γ ELISPOT assay determine whether T cells derived from patients with ovarian cancer would recognise ovarian TAAs and produce IFN-γ in response to priming with autologous DCs preloaded with oxidised SK-OV-3.

- To demonstrate the presence of HER-2/neu specific T cells after priming with patients' autologous DCs pulsed with oxidised SK-OV-3.

- To assess the ability of cancer patients' DCs to mature in the presence of two maturation stimuli, i.e. agonistic CD40 mAb and MPL, and to compare the T cell stimulatory capacity of such mature DCs to immature DCs preloaded with HOCl-oxidised SK-OV-3 cells in cross-priming T cells *in vitro*.

- To evaluate the ability of patients' T cells primed with mature DCs (treated with agonistic CD40 mAb or MPL) preloaded with oxidised SK-OV-3 in recognising autologous ascites in the absence of APCs in IFN-γ ELISPOT assay.

- To investigate whether patients' T cells stimulated with mature DCs loaded with oxidised tumour cells were immunogen and tissue-specific.

4.3. Results

4.3.1. T cells from patients with ovarian cancer are not tolerant to autologous dendritic cells pulsed with oxidised SK-OV-3 tumour cells

Previously it was showed that DCs, derived from HLA-$A2^+$ healthy volunteers and preloaded with oxidised SK-OV-3, stimulated T cells that recognised the immunising antigen (i.e. oxidised SK-OV-3) and unmodified (i.e. not oxidised) peptides derived from HER-2/neu and MUC1, two TAAs expressed by the SK-OV-3 cell line (Chiang *et al.*, 2006). In this present study, the responses of seven healthy volunteers was compared to the responses from a group of ten patients with ovarian cancer using DCs loaded with oxidised SK-OV-3 and autologous T cells (Fig. 4.1A). All ten patients responded to the oxidised cells (>500 spots per million). The average number of spots was lower than in the volunteer cohort ($P< 0.01$, MannWitney test), which might reflect differences in age distribution and general health. Indeed the mean background (media alone, Fig. 4.1B) response of the healthy volunteers was also slightly higher than that of the patients, although this difference was not statistically significant. A typical dose response to the oxidised SK-OV-3 in a representative patient is shown in Fig. 4.1C.

Four HLA-$A2^+$ individuals from this group were also tested using HLA-A2 restricted HER-2/neu and MUC1 peptides, and a control peptide derived from the melanoma antigen MART-1 (Fig. 4.1D). Strong responses were seen to all four HER-2/neu and MUC1 peptides tested but there was no response to the MART-1 peptide. Since the SK-OV-3 line is HLA-$A2^-$, these experiments demonstrated that oxidised SK-OV-3 cells were cross-presented by the DCs, and that the T cells recognised non-oxidised TAAs. All four patients expressed high levels of tumour MUC1 as assessed by

immunocytochemistry, and one (patient 27) was also positive for HER-2/neu (Table 4.1). No responses were seen to either oxidised SK-OV-3 cells or peptides when PBMCs were tested directly from patients, without DC presentation/expansion, thus confirming that exposure to the tumour *in vivo* had not primed a significant response. It was noted that an important control that was absent was to determine the precursor frequency of ovarian-specific T cells in naïve unprimed T cell population in IFN-γ ELISPOT. This data would be useful for determining whether *in vitro* priming of T cells with oxidised SK-OV-3 would indeed increase the number of such tumour-specific T cells. This was not done due to the limitation of the number of patients' T cells obtained and thus all the T cells were used for *in vitro* priming experiment.

As an additional measure of antigen specificity, pentamer staining was performed using a HER-2/neu peptide (E75; KIFGSLAFL)/HLA-A2 pentamer (Fig. 4.2). After 3 weeks of *in vitro* stimulation (i.e. priming with oxidised SK-OV-3 loaded DCs, and expansion with HER-2/neu peptide), between 2 and 4% of the $CD8^+$ T cells were specific for the HER-2/neu pentamer. These results were very similar to those obtained previously from healthy volunteers (Chiang *et al.*, 2006). Less than 0.2% pentamer specific cells were detected on T cells expanded on a MUC1 peptide (data not shown). All three patients' tumours were MUC1 positive, and one patient's tumour was also positive for HER-2/neu expression. An important control which was lacking was the HER-2/neu pentamer staining of naïve unprimed T cells to determine the precursor frequency of ovarian-specific T cells, and for comparison after antigen stimulation. This was not done due to the limitation of the number of patients' T cells that was available for this assay and the IFN-γ ELISPOT that was set up in parallel.

4.3.2. Ovarian cancer patients' dendritic cells matured normally when stimulated with CD40 agonistic antibody or monophosphoryl lipid A

Our previous study showed that oxidised SK-OV-3 cells induced only a partial maturation of DCs. Thus in this study DCs were matured further by addition of anti-CD40 antibody, or the TLR4 agonist MPL. The optimum concentration was first determined by monitoring DC maturation by flow cytometry. As shown in Fig. 4.3 (left panels), upregulation of DC maturation markers and costimulatory molecules – CD83, CD86, HLA-DR and CD40 – was observed with increasing doses of CD40 antibody or MPL. The relatively low expression of CD40 observed on CD40-activated cells may reflect competitive interference between the antibody used to activate the cells and the antibody used to monitor expression, although two different mAb antibodies were used. The viability of DCs was reduced by 10% at 1000 ng/ml CD40 agonistic antibody treatment (data not shown). Representative fluorescence histograms and median fluorescence values are shown in the right panels of Fig. 4.3 for DCs that had been loaded with oxidised tumour cells in the absence of further stimulus, in the presence of MPL (100 ng/ml) or in the presence of CD40 antibody (500 ng/ml), the concentrations that were used for further experiments.

****

Table 4.1 Details of patients used in the study

Patient no.	HLA-A2 status	Age	Tumour histology	Tumour grade	MUC-1 staining		HER-2/neu staining
					Pattern	Intensity	
1	Positive	70	Serous	3	Cyto, mem	3+	0
6	Positive	58	Serous	3	NA	NA	NA
7	Positive	70	Serous	2	Cyto, mem	3+	3+ ■
8	Negative	64	Serous	2	Cyto, mem	3+	1+
9	Positive	52	Clear cell	NA	NA	NA	NA
12	Positive	62	Serous	2	Cyto, mem	3+	0
13	Negative	77	Endometrioid	2	Cyto, mem	3+	3+ ■
14	Negative	52	Endometrioid	2	Cyto, mem	2+	3+ ■
15	Negative	63	Serous	3	Cyto, mem	3+	0
16	Not done	68	Endometrioid	3	NA	NA	NA
17	Negative	63	Serous	2	Cyto.mem	3+	2+
18	Negative	67	Serous	3	Cyto, mem	2+	3+ ■
19	Negative	67	Endometrioid	2	Cyto	3+	0
20	Negative	60	Serous	2	Cyto, mem	3+	1+
21	Not done	58	Endometrioid	2	Cyto, mem	3+	1+
22	Not done	48	Serous	3	Cyto, mem	3+	0
23*	Negative	70	Serous	3	Cyto, mem	3+	0
24	Not done	46	Serous	3	NA	NA	NA
26*	Positive	72	Endometrioid	2	Cyto, mem	3+	1+
27	Positive	60	Serous	3	Cyto, mem	3+	2+

Note[1]: Cyto- cytoplasm; mem – membrane; Endometrioid – endometrioid carcinoma; Serous – serous carcinoma; Clear cell – clear cell carcinoma; Inv duc Breast – invasive ductal breast carcinoma

NA: no tissue sample available for these patients; * ascites also available and tested; ■ HER-2/neu 3+ interpreted as a clear positive

Note[2]: Patient 3, 4, 5, 6 and 10 were not shown in the table as they were used for another study, while Patient 2, 11 and 25 gave insufficient number of cells for experiments.

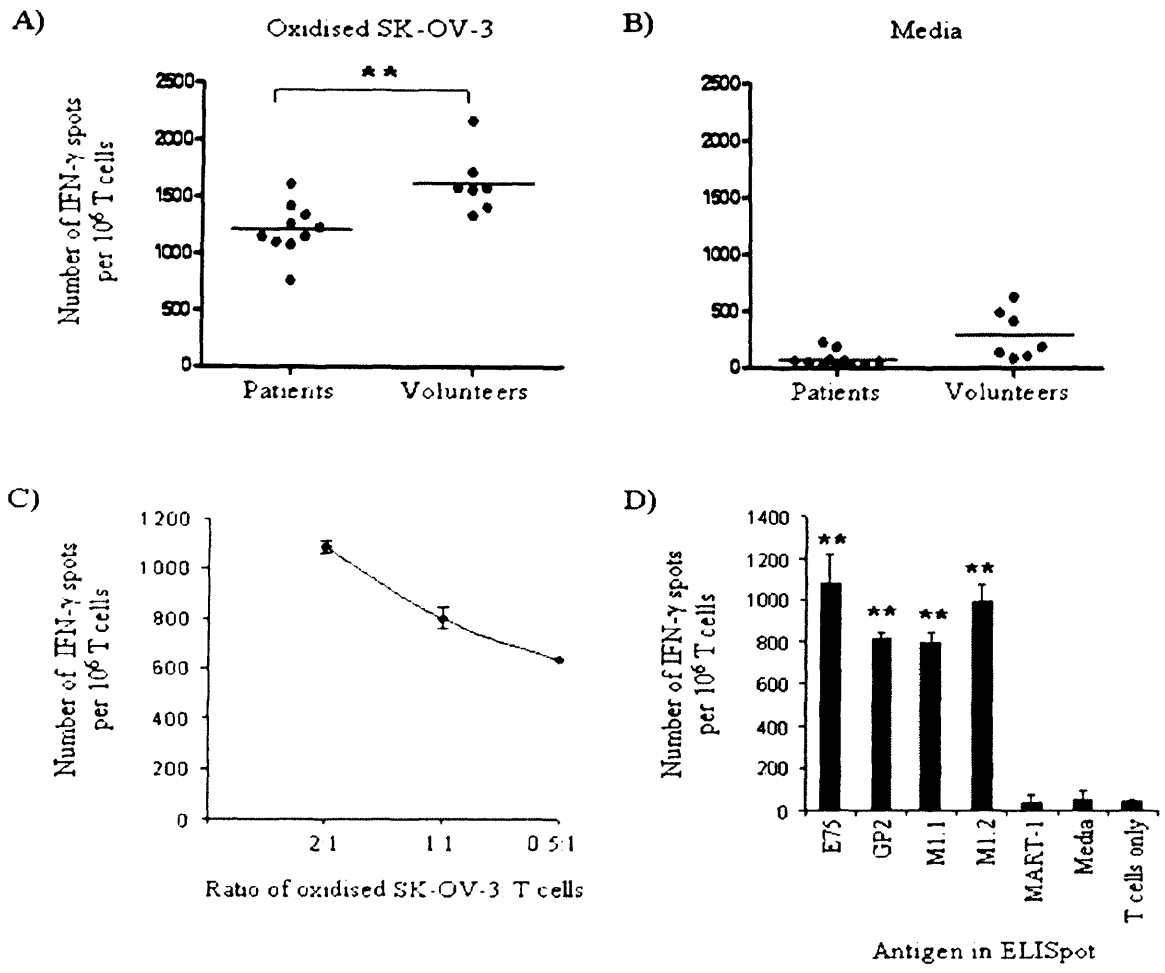


Figure 4.1 T cells from patients with ovarian cancer respond to autologous dendritic cells loaded with oxidised SK-OV-3 cells. IFN-γ T cells responses using T cells and DCs isolated from ten patients and seven healthy volunteers. DCs were cocultured with A) oxidised SK-OV-3 or B) complete AIM-V media and then with autologous T cells. After a two week expansion period, T cells were collected and restimulated with oxidised SK-OV-3 cells (ratio of 1 T cell: 1 tumour cell) and PBMCs in ELISPOT assays. Each point represents the average of triplicate cultures from a different individual. The line shows the median response. The two groups were compared using Mann Whitney. The difference between the responses of patients' and volunteers' T cells to oxidised SK-OV-3 was statistically significant (**, $P < 0.01$; Mann-Whitney), and both these responses were significantly greater than to media (**, $P < 0.01$; Mann-Whitney). C) T cells from a HLA-$A2^-$ patient were expanded with oxidised SK-OV-3 loaded DCs as in A, and restimulated with PBMC and different ratios of oxidised SK-OV-3 cells as shown. The figure shows the average and standard error of triplicate cultures from one representative individual out of ten. D) T cells from HLA-$A2^+$ patients were expanded with oxidised SK-OV-3 loaded DCs as in A, and restimulated with PBMC and HER-2/neu (E75 and GP2), MUC1 (M1.1 and M1.2), MART-1 or media control in ELISPOT assays. The last 2 columns show the response of T cells in the absence of antigen or PBMCs. The figure shows mean and standard error of responses from four individuals. The mean differences between the responses of T cells to the peptides (i.e. E75, GP2, M1.1, M1.2) compared to media were statistically significant (**, $P < 0.01$; Anova with Dunnetts post-hoc modification).

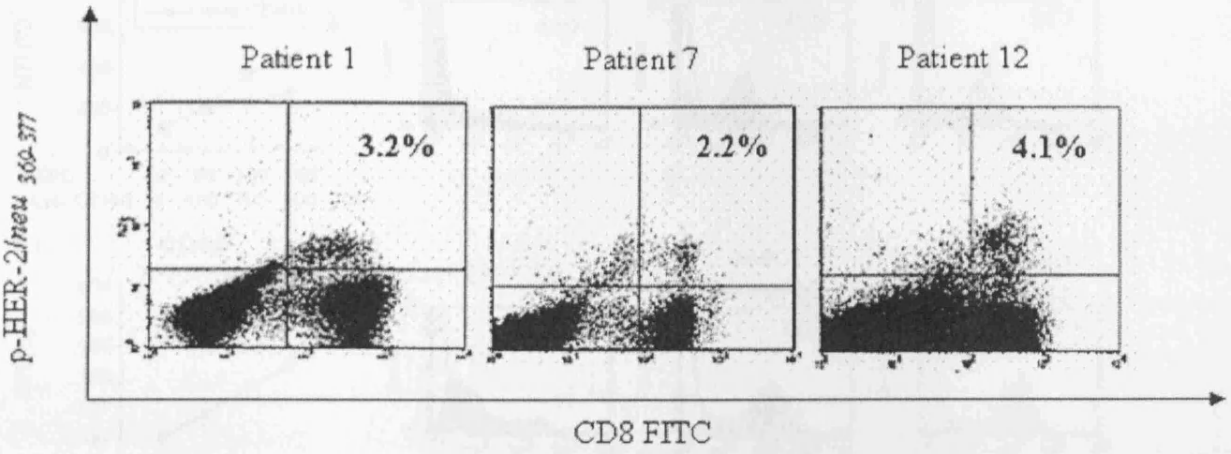


Figure 4.2 Patients' dendritic cells preloaded with oxidised SK-OV-3 stimulated HER-2/neu specific T cells. DCs were cocultured with oxidised SK-OV-3 and then with autologous T cells as described in Materials and Methods. After a three week expansion period, T cells were collected, and stained with HER-2/neu (HLA-A2/ KIFGSLAFL epitope) pentamers and CD8-FITC. The number in the upper left panel indicates the percentage pentamer positive cells as a proportion of total CD8+ cells. The figure shows results obtained from three individuals (patient numbers refer to Table 4.1).

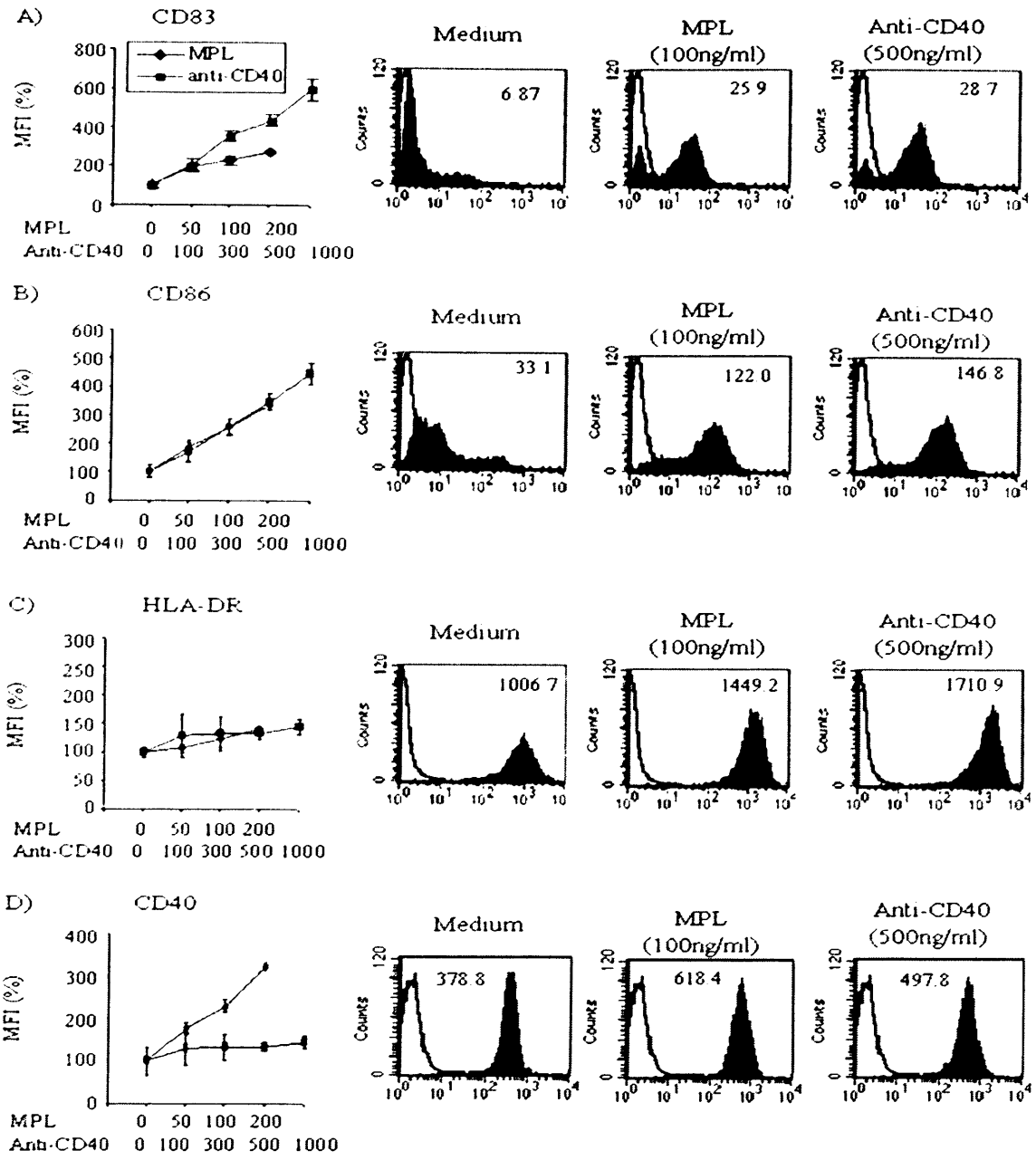


Figure 4.3 Activating anti-CD40 and MPL induce further maturation in patients' dendritic cells loaded with oxidised SK-OV-3 cells. DC were prepared from patient PBMCs and cultured in the presence of different concentrations of MPL (50-200 ng/ml) or CD40 agonistic antibody (100-1000 ng/ml) for 24 h. Expression of each marker was measured independently by flow cytometry using PE-conjugated antibodies as described in Materials and Methods. The left panel shows the average mean fluorescent intensity (MFI) ± standard error bars from three independent experiments, expressed relative to the MFI in the absence of stimulus (defined as 100%). The right panel shows one representative frequency histogram of fluorescence (FL2 channel) in the absence of stimulus, or in the presence of 100 ng/ml MPL or 500 ng/ml CD40 agonistic antibody. The number in the right hand top corner of each histogram shows the MFI (channel number). The empty profile shows negative control staining with control PE-conjugated IgG.

4.3.3. Mature dendritic cells loaded with oxidised SK-OV-3 induced strong $CD4^+$ and $CD8^+$ tumour-specific responses

Two experimental approaches were used to demonstrate a $CD4^+$ T cell response. In Fig. 4.4, unfractionated T cells were tested against a range of ovarian antigens in the ELISPOT, including two HER-2/neu peptides that had previously been shown to represent promiscuous MHC class II epitopes that bound to a wide variety (though not all) of HLA-DR haplotypes (Baxevanis *et al.*, 2006). Immature DCs were compared to DCs matured by CD40 agonistic antibody (as detailed above) for these experiments. Unfractionated T cells stimulated by immature DCs gave good responses to oxidised SK-OV-3 cells (as above), and these responses were further boosted (about two fold) by using mature DCs (Fig. 4.4A). In contrast, T cells failed to respond ($P > 0.5$, response not significantly above background) to either of the MHC class II peptides when primed by immature DCs (Fig. 4.4C, D). However, priming with mature DCs loaded with oxidised SK-OV-3 stimulated responses against both the class II HER-2/neu peptides tested, H360 and H776 [both $P < 0.01$ when compared to priming with immature DCs] (Fig. 4.4C, D).

In a second approach (Fig. 4.5), T cells were depleted of $CD8^+$ T cells before coculturing with DCs. After depletion, the $CD4^+$ T cell population which contained about 5% contaminating $CD8^+$ T cells was tested in the assay. The total $CD4^+$ T cell population gave a smaller, but significant response to oxidised SK-OV-3 cells, which was enhanced about 2-3 times by using mature DCs [in these experiments matured with MPL] (Fig. 4.5A). Neither immature nor mature DCs primed responses to peptide H369 (Fig. 4.5C), while mature DC primed a small response to H776 (Fig. 4.5D).

4.3.4. Dendritic cells pulsed with oxidised SK-OV-3 primed IFN-γ producing T cells that efficiently recognise autologous ovarian tumour cells isolated from ascites The key test for the strategy of using oxidised SK-OV-3 cells as a generic cell based antigen is whether T cells primed in this way will recognise autologous tumour. For these experiments PBMC were taken from two patients in remission, from whom ascitic tumour cells had been collected and stored in advance in -80°C. The expression of HER-2/neu and MUC1 in the tumour sample itself was analysed by immunoblotting (Fig. 4.6A and B). Tumour MUC1 glycoproteins were detected as multiple glycosylated variants of approximately 250 kDa in the SK-OV-3 ovarian cell line and in both patients' ascites samples. HER-2/neu (molecular weight approximately 185 kDa) was detected as a single band in the SK-OV-3 line but was absent in both ascites samples. Both antigens were absent from the melanoma tumour line MEL-11. It was noted that dilution controls for both HER-2/neu and MUC1 proteins (i.e. loading fewer tumour cells or less tumour proteins per lane) were not performed. The data would be useful to show clearer and more defined bands, especially for the MUC1 protein, in the western blot.

For functional studies, on the basis of the data shown in Figs. 3 and 4, we used DCs loaded with oxidised SK-OV-3 and then cultured with either CD40 agonistic antibody or MPL. The first patient (patient 23 in Table 1) was HLA-A2$^-$, and therefore was tested with the promiscuous class II MHC restricted peptides, but not the HLA-A2 restricted peptides (Fig. 4.7A). The T cells responded to both MHC class II peptides tested (Fig. 4.7A), as well as to the oxidised SK-OV-3 used for priming (Fig. 4.7B). Critically, the T cells primed with oxidised SK-OV-3 also showed responses to the autologous unmodified tumour cells over a range of tumour to T cell ratios, and in the

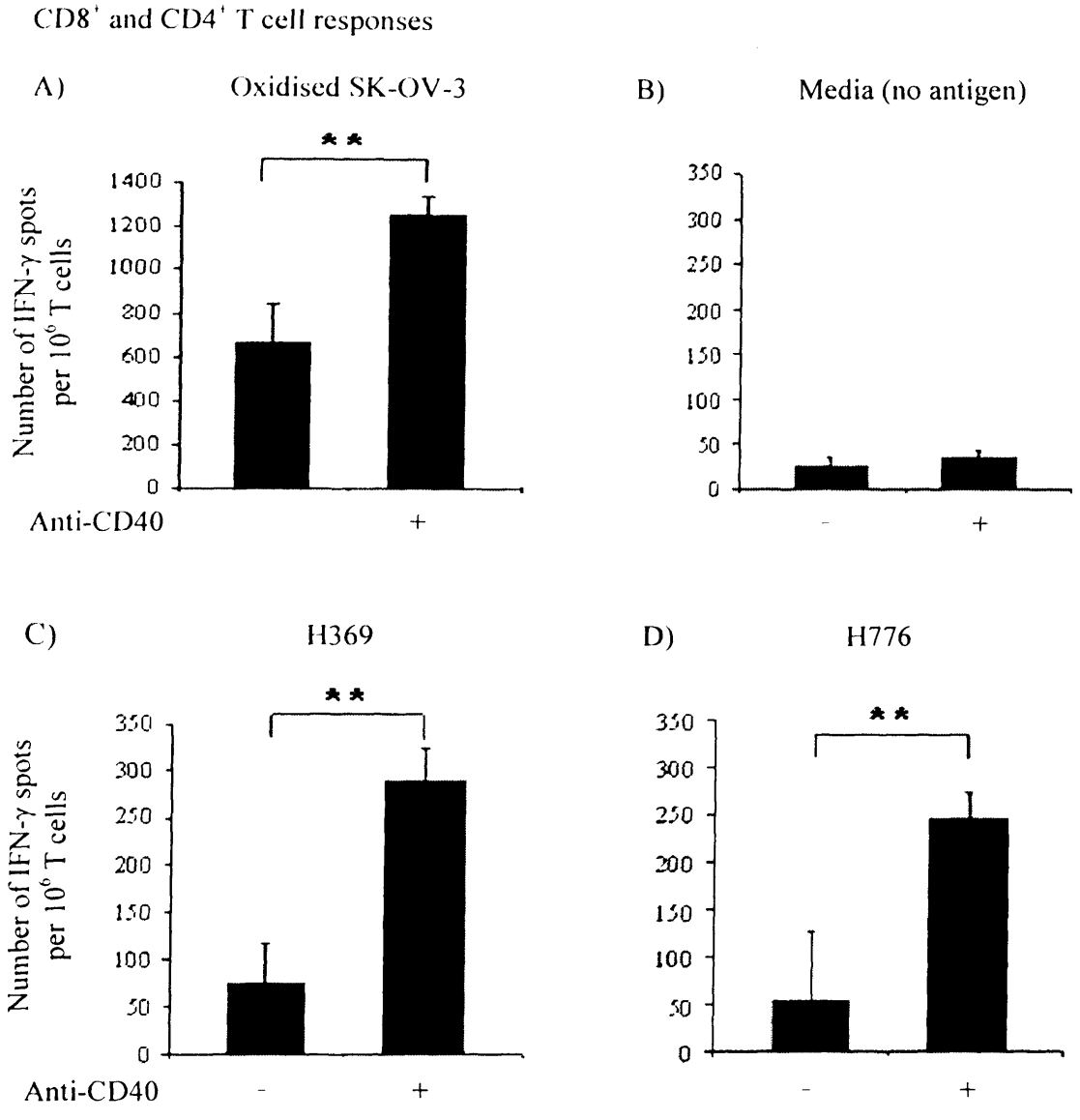


Figure 4.4 Dendritic cells matured with agonistic CD40 antibody induce $CD4^+$ as well as $CD8^+$ tumour-specific T cell responses. Unfractionated T cells isolated from patient PBMCs were cocultured with autologous DCs that were preloaded with oxidised SK-OV-3, and then either matured with 500 ng/ml CD40 agonistic antibody or cultured in complete AIM-V media alone. After a 2-week expansion period, T cells were isolated and tested in IFN-γ ELISPOT assays with PBMCs (1×10^4) and oxidised SK-OV-3 cells (ratio of 1 T cell : 1 tumour cell), or HER-2/neu class II MHC peptides H369 and H776 (all at a final concentration of 1μM), or complete AIM-V media only. The results show mean number of spots per million T cells from four independent experiments (i.e. different patient PBMCs in each experiment). The T cell responses to all antigens tested (i.e. oxidized SK-OV-3, H369 and H776) using anti-CD40 matured DCs were statistically greater (**, $P < 0.01$, Student's paired *t*-test) than those using dendritic cells without anti-CD40, and also significantly greater ($P < 0.05$; Anova with Dunnetts post-hoc modification) than those to responses in the absence of antigen.

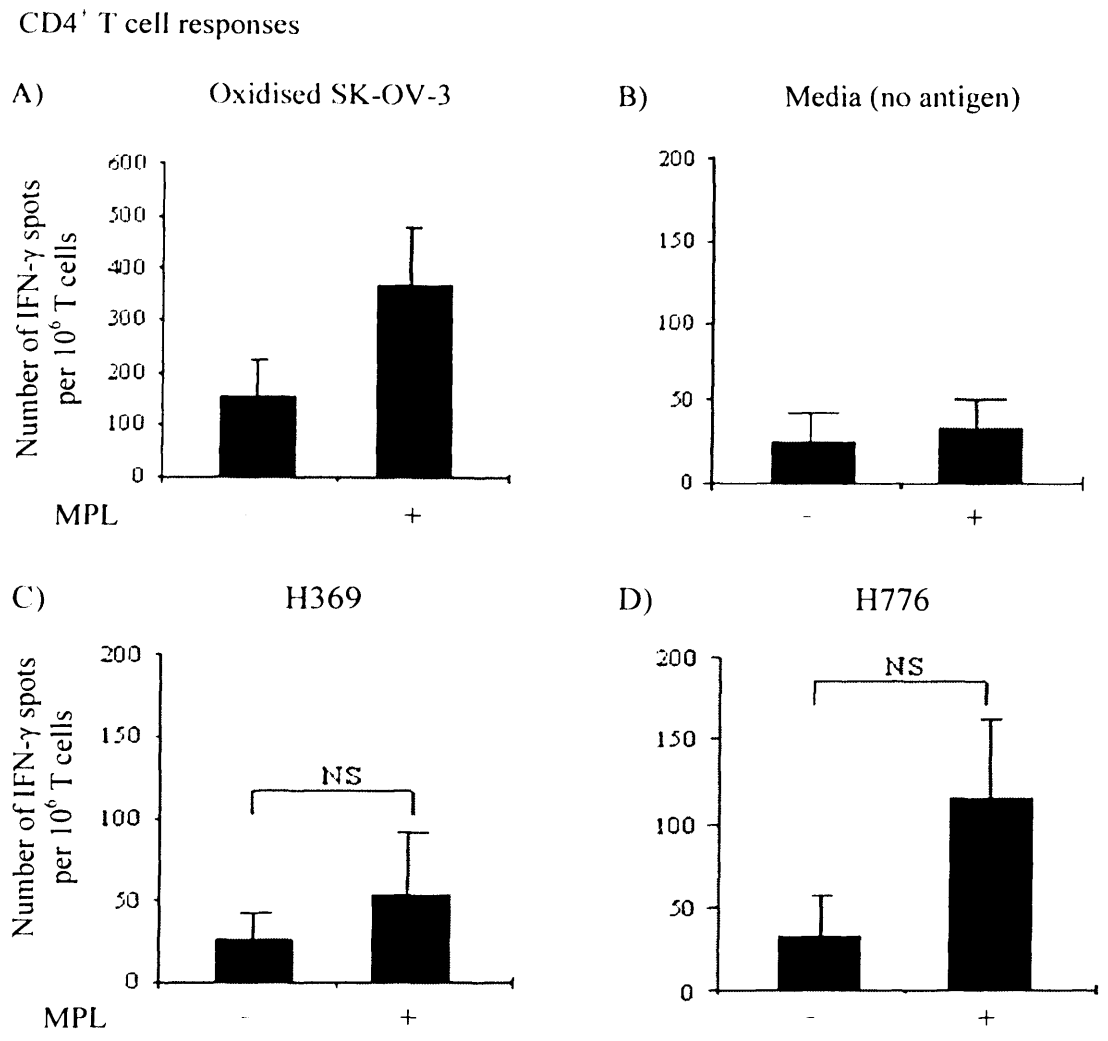


Figure 4.5 Dendritic cells matured with MPL enhance $CD4^+$ tumour-specific T cell responses. T cells isolated from patient PBMCs were depleted of $CD8^+$ T cells by immunomagnetic bead selection as described in Materials and Methods. The residual T cells contained about 5% $CD8^+$ T cells. The purified $CD4^+$ cells were cocultured with autologous DCs that were preloaded with oxidised SK-OV-3, and then either matured with 100 ng/ml MPL or cultured in complete AIM-V media alone. After a 2-week expansion period, viable $CD4^+$ T cells were isolated and assessed in IFN-γ ELISPOT assays with PBMCs (1×10^4) and oxidised SK-OV-3 cells (ratio of 1 T cell : 1 tumour cell), or HER-2/neu class II MHC peptides H369 and H776 (all used at a final concentration of 1μM), or complete AIM-V media only. The results show mean number of spots per million T cells from three independent experiments (i.e. different patient PBMCs in each experiment). T cell responses from the MPL treatment were statistically significant compared to that of no MPL treatment in the oxidised SK-OV-3 group (*, $P < 0.05$; Student's paired *t*-test) but not for H369 and H776 groups (*NS*, not significant). The response to oxidised SK-OV-3 and H776 with MPL treatment was statistically significant ($P < 0.05$; Anova with Dunnetts post-hoc modification) compared to no MPL treatment and no antigen.

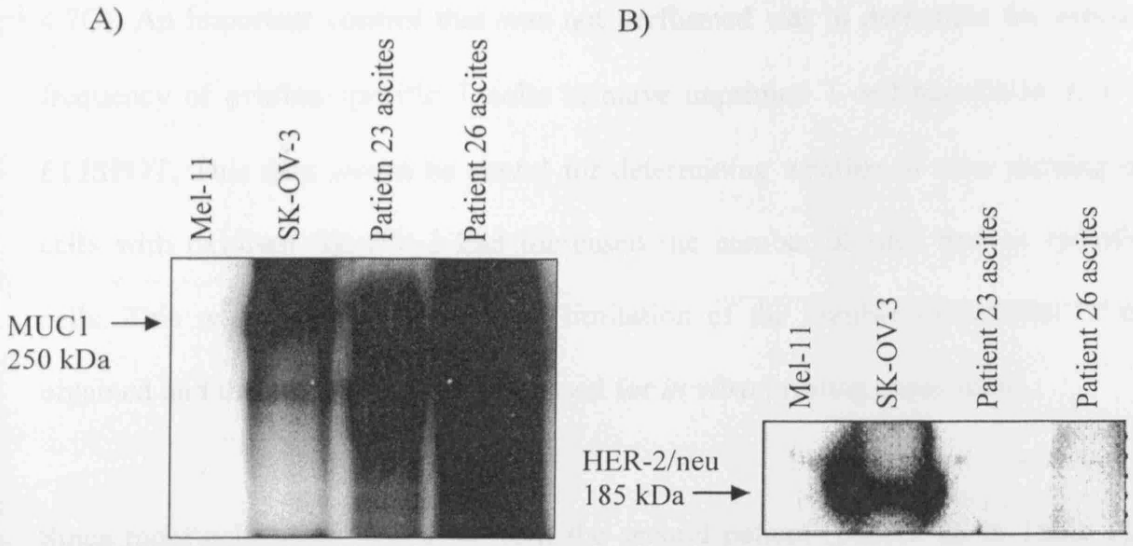


Figure 4.6 Expression of MUC1 and HER-2/neu tumour antigens on ovarian cancer patients' ascites.1 x 10^6 ascite tumour cells, SK-OV-3 cells or MEL-11 cells were lysed in sample buffer, resolved on 7.5% SDS-PAGE electrophoresis, and transferred to nitrocellulose membranes as described in Materials and Methods. MUC1 and HER-2/neu expressions were detected by anti-MUC1 and anti-HER-2/neu monoclonal antibodies, respectively. In A) MUC1 glycoproteins were detected as multiple glycosylated variants of approximately 250 kDa in the ascite tumour cells of Patient 23 and 26, and in SK-OV-3. In B) HER-2/neu appeared as a single band of 185 kDa in the SK-OV-3 but absent in the patients' ascites. MEL-11 was negative for both MUC1 and HER-2/neu.

absence of any exogenous antigen presenting cells in the ELISPOT assays (Fig. 4.7C). An important control that was not performed was to determine the precursor frequency of ovarian-specific T cells in naïve unprimed T cell population in IFN-γ ELISPOT. This data would be useful for determining whether *in vitro* priming of T cells with oxidised SK-OV-3 had increased the number of such tumour-specific T cells. This was not done due to the limitation of the number of patients' T cells obtained and thus all the T cells were used for *in vitro* priming experiment.

Since more cells were available from the second patient (patient 26 in Table 1), an additional specificity control was added. Half the DCs were loaded as above with oxidised SK-OV-3 cells, while half were loaded with an oxidised melanoma line, MEL-11 (also HLA-A2$^-$). Since the individual was HLA-A2$^+$, the T cells responded to the class I HER-2/neu peptide E75, as well as the class II MHC HER-2/neu peptides tested (Fig. 4.8A). There was no response to the melanoma HLA-A2 restricted peptide MART-1. The T cells stimulated with the oxidised SK-OV-3 cells also responded to oxidised SK-OV-3 cells, but showed no response to oxidised MEL-11 cells (Fig. 4.8B). As with patient 23, the T cells also reacted to unmodified autologous tumour in the absence of exogenous APCs (Fig. 4.8C), demonstrating directly that they were not tolerant to the tumour cells. In contrast, the T cells stimulated *in vitro* with the oxidised MEL-11 cells showed little response to the autologous ovarian tumour cells (Fig. 4.9A). The cells did respond strongly to the oxidised MEL-11 cells, and showed a significant response to the MART-1 peptide, but no response to any HER-2/neu peptide tested (Fig. 4.9B). Thus the T cells stimulated by oxidised antigen remain tolerant to common self antigens shared

Patient 23 (HLA-A2 negative)

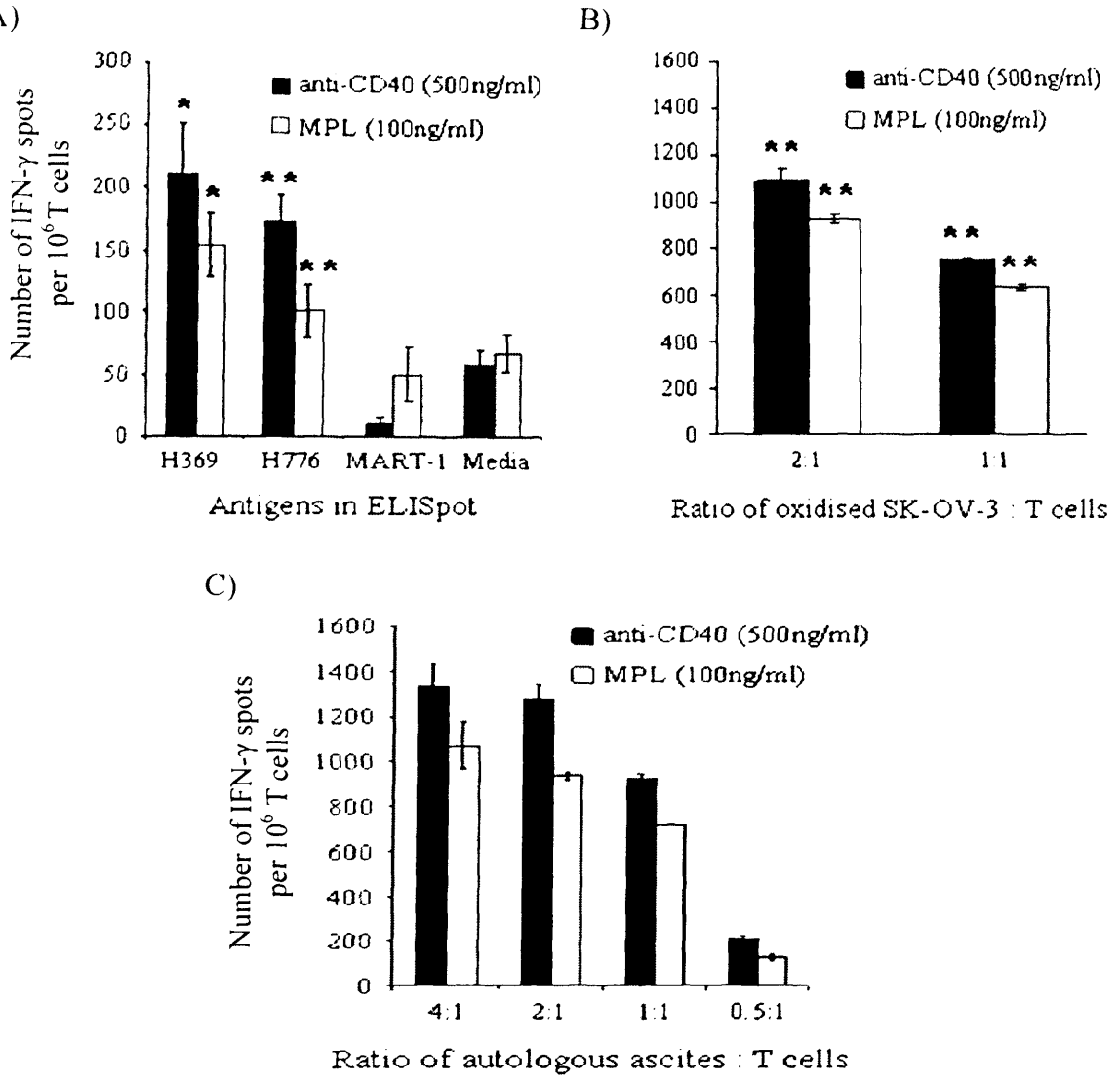


Figure 4.7 Patient's T cells stimulated with mature dendritic cells pulsed with oxidised SK-OV-3 efficiently recognise autologous ascites derived tumour cells and ovarian tumour-associated antigens. T cells from a HLA-A2 negative patient (Patient 23) were cocultured with autologous DCs that were loaded with oxidised SK-OV-3, and then matured with CD40 agonistic antibody (500 ng/ml) or MPL (100 ng/ml). After a 2-week expansion period, T cells were isolated and tested in IFN-γ ELISPOT assays. In A) T cells were cocultured with PBMCs (1 x 10^4) and HER-2/neu class II MHC peptides H369 and H776 , or control peptide MART-1 (all at a final concentration of 1 μM), or complete AIM-V media only. In B) T cells were cocultured with PBMCs (1 X 10^4) and oxidised SK-OV-3 cells (ratios of SK-OV-3: T cells were 2:1 or 1:1 as shown). In C) T cells were cocultured in the absence of PBMCs with autologous ascites-derived tumour cells at various ratios as shown. The results show mean number of spots per million T cells, from triplicate cultures. *P* value is significant if < 0.05 (*) or < 0.01 (**) when comparing the test groups to the media control using Anova with Dunnetts post-hoc modification. In panel C asterisks are not shown but all columns are significantly different from media control (p <0.01 or p <0.05).

Patient 26 (HLA-A2 positive)

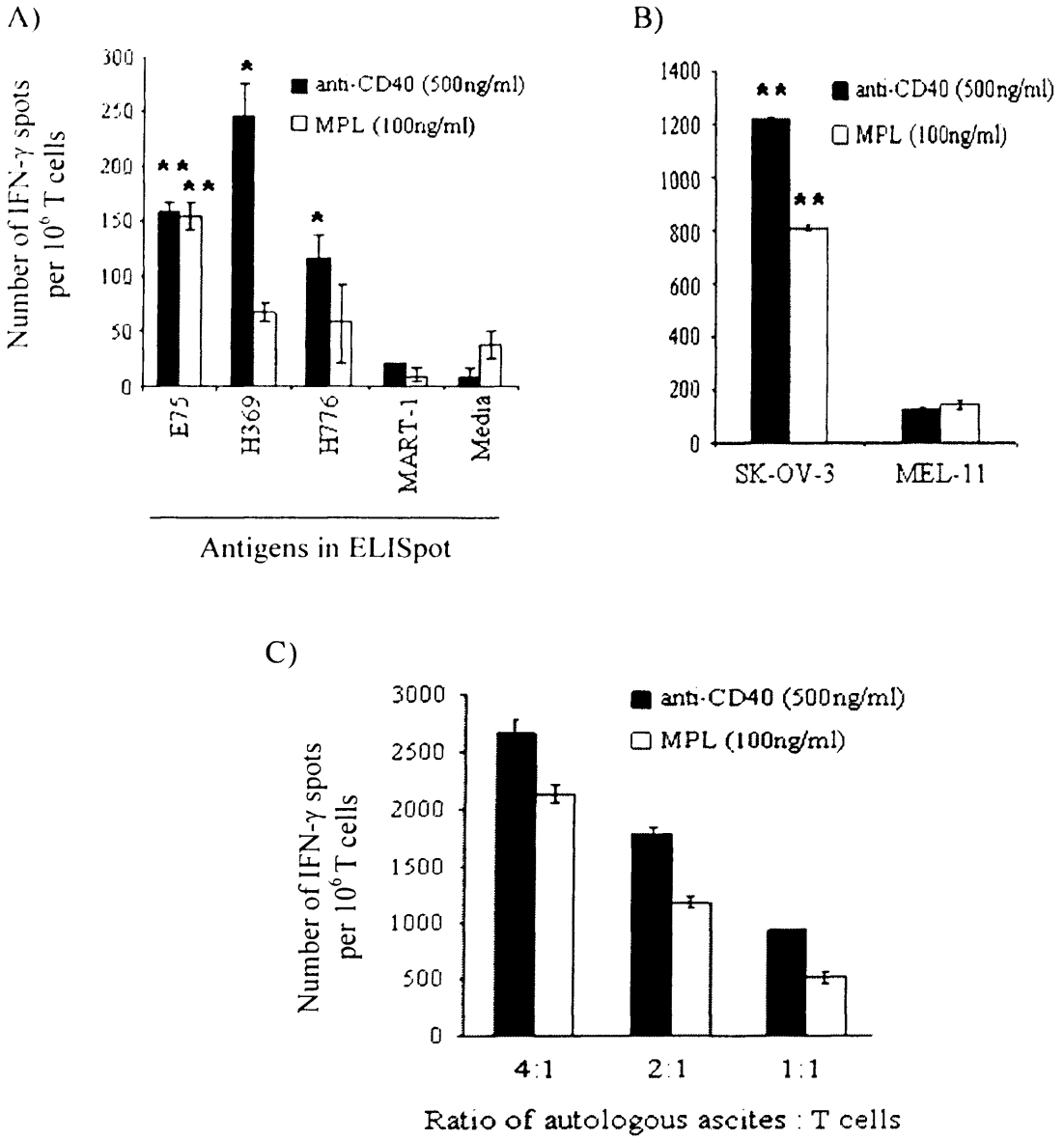
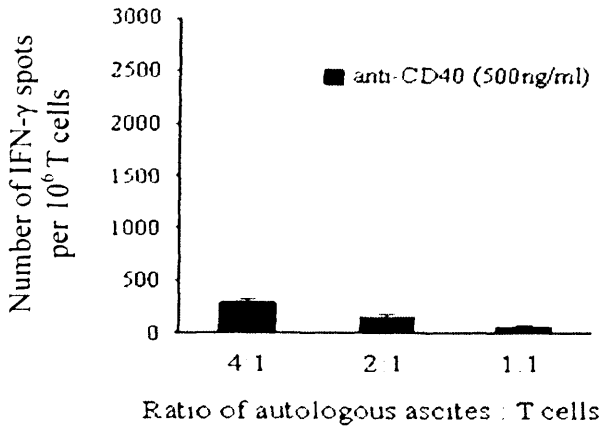


Figure 4.8 Patients' T cells stimulated with mature dendritic cells pulsed with oxidised SK-OV-3 cells demonstrate tumour-specificity. T cells from a HLA-$A2^+$ patient (Patient 26) were co-cultured with autologous DCs preloaded with oxidised SK-OV-3 and matured with CD40 agonistic antibody (500 ng/ml) or MPL (100 ng/ml). After a 2-week expansion, T cells were isolated and evaluated in IFN-γ ELISPOT assays. In A) T cells were cocultured with PBMCs (1 x 10^4) and HER-2/neu class I MHC (HLA-A2, E75) and HER-2/neu class II MHC peptides H369 and H776, or control peptide MART-1 (all at a final concentration of 1μM), or complete AIM-V media only. In B) T cells were cocultured with PBMCs (1 x 10^4) and oxidised SK-OV-3 cells or oxidised MEL-11 (ratio of 1 tumour cell: 1 T cells). In C) T cells were cocultured in the absence of PBMCs with autologous ascites-derived tumour cells at various ratios as shown. *P* value is significant if < 0.05 (*) or < 0.01 (**) when comparing the test groups to the media control using Anova with Dunnetts post-hoc modification.

Patient 26 (HLA-A2 positive)

A)



B)

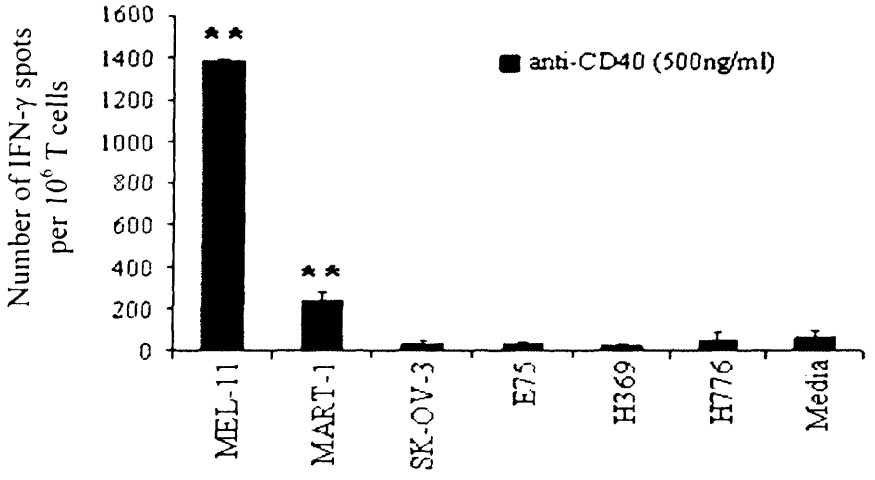


Figure 4.9 Patients' T cells stimulated with mature dendritic cells loaded with oxidised MEL-11 cells were immunogen-specific and did not recognise autologous ascites. T cells from a HLA-$A2^+$ patient (Patient 26) were cocultured with autologous DCs that were loaded with oxidised melanoma cell and then matured with CD40 agonistic antibody (500 ng/ml). After 2 weeks of stimulation, T cells were isolated and assessed in IFN-γ ELISPOT assays. In A) T cells were cocultured in the absence of PBMCs with autologous ascites-derived tumour cells at various ratios as shown. In B) T cells were cocultured with PBMCs (1×10^4) and oxidised MEL-11 or oxidised SK-OV-3 cells (ratio of 1 tumour cell: 1 T cell), or various peptides as detailed above. The results show mean number of spots per million T cells, from triplicate cultures. *P* value is significant if < 0.05 (*) or < 0.01 (**) when comparing the test groups to the media control using Anova with Dunnetts post-hoc modification.

between cells of different tissue origin, but can respond to tissue specific antigens expanded *in vitro* by DCs loaded with SK-OV-3 or MEL-11, respectively.

4.4. Discussion

In this study we have shown that the oxidised tumour line SK-OV-3 can induce DC-mediated stimulation of antigen specific T cells isolated from ovarian cancer patients. This priming, similar albeit lower to that seen previously in healthy controls, was a very consistent feature in all patients examined. The studies presented in chapter 3 have already demonstrated that non-oxidised tumour cells either live or killed by non-oxidative means have very poor stimulatory activity in this system (Chiang *et al.*, 2006), and these antigens were therefore not explored further here.

The responses seen included both MUC1 and HER-2/neu epitopes (even though the intrinsic tumours overexpressed one or both these antigens) and were elicited from both $CD8^+$ and $CD4^+$ T cells. T cell priming resulted from cross-presentation, since although SK-OV-3 is HLA-$A2^-$, HLA-$A2^+$ DCs presenting oxidised SK-OV-3 cells stimulated T cell responses to HLA-A2 restricted peptides. Furthermore the T cells stimulated by cross-presentation of oxidised SK-OV-3 cells recognised autologous tumour cells isolated from ascites. Thus we propose that oxidised tumour cell lines may be very useful tools as potent generic immunogens for DC immunotherapy protocols that aim to generate T cells which can target autologous tumour.

The model sheds light on some key questions regarding the tolerance/effector balance, which are fundamental both to our understanding of tumour immunology and to advancing realistic prospects for successful DC driven adoptive immunotherapy. An

important objective of the study was to demonstrate that presentation of oxidised SK-OV-3 cells could breach *in vivo* tolerance to tumour i.e. that at least a proportion of the T cells stimulated by DCs loaded with oxidised SK-OV-3 were in fact directed at bona fide "self" tumour antigens to which the immune system had previously been exposed *in vivo*. Oxidised SK-OV-3 cells contain a variety of non-self antigens, including both SK-OV-3 specific alloantigens (since this line is not syngeneic to the volunteers or the patients used in this study) and neo-epitopes formed by the oxidation process. Responses against bona fide tumour antigens were therefore documented in two ways.

Firstly, ovarian cancers often overexpress a variety of TAAs, including the well characterised HER-2/neu (Hellstrom *et al.*, 2001) and MUC1 (McGuckin *et al.*, 1995) proteins, as confirmed in the responding individuals for this study (see Table 4.1). A panel of peptides encoding known MHC class I and MHC class II peptides from these antigens were recognised by the oxidised SK-OV-3 *in vitro* stimulated T cells. This implies cross-presentation of SK-OV-3 proteins by the DCs via both class I and class II MHC, and establishes clearly that any prior exposure did not prevent the ability of the SK-OV-3 loaded DCs to stimulate T cell responses to defined tumour antigen epitopes. Secondly, T cells stimulated by the oxidised SK-OV-3 cells recognised and responded strongly to autologous unmodified tumour cells, in the absence of any DCs (Figs 4.7C and 4.8C). Although we have so far been able to collect matched PBMCs and ascites from only two individuals, these results suggested that at least in a proportion of individuals, the oxidised SK-OV-3 share sufficient antigens with primary ovarian tumours to act as a generic antigen for DC immunotherapy.

Furthermore, presentation of the oxidised cells by DCs is sufficient to break any existing tolerance to these primary ovarian cancer-related antigens at least *in vitro*.

Since the immune system is exposed to tumour antigens in large amounts *in vivo* (especially in the case of development of a large bulk of coelomic cavity metastatic tumour, associated with ascites), but this does not prevent growth of tumour, it is clear that an efficient effector response does not occur in these patients. Many mechanisms may contribute to tumour escape, including failure to prime an effector response, active tolerance induction, and tumour evasion of effector mechanisms. We were unable to detect any *ex vivo* responses to tumour cells in patient PBMCs without prior *in vitro* stimulation (data not shown). Furthermore, the *in vitro* response stimulated by DCs loaded with oxidised SK-OV-3 cells was not amplified in patients versus volunteers. Both these observations suggest that if there is any effector T cell immune response stimulated *in vivo*, at least to MUC1 and HER-2/neu then it is very limited.

A second important observation concerned the ability of oxidised cellular antigen to activate tumour antigen specific T cells in a class II as well as a class I MHC restricted fashion. There is considerable evidence which suggests that $CD8^+$ T cell responses primed in the absence of cognate help are impaired functionally in a variety of ways (Chamoto *et al.*, 2006; Nishimura *et al.*, 1999; Xiang *et al.*, 2005; Zhang *et al.*, 2007). Our findings indicate that the *in vitro* protocol adopted here can overcome these limitations. Both by using two peptide epitopes known to bind a broad range of class II MHC molecules, and by direct depletion of $CD8^+$ T cells, we showed that class II MHC restricted $CD4^+$ T cell responses are activated in these cultures. The response to the peptides is variable, and may reflect differential binding of these

peptides to different HLA alleles (note that we did not HLA type the individual patients, other than to determine if they were HLA-A2 positive or negative). The overall magnitude of the response is much smaller (at least in terms of IFN-γ producing cells per million T cells) than the class I response, a difference which is common for immune responses to many viral antigens (Maini *et al.*, 2000). Furthermore, the response was more dependent on full maturation of the DCs, perhaps reflecting the greater dependency of the $CD4^+$ T cell response on costimulatory activity by the APCs.

The third important observation in this study was the tumour specificity of the response. Given that the effectiveness of tumour therapy must depend on partial breaking of self tolerance, a persistent concern has been that the immune system will recognise and kill cells other than the tumour and hence cause autoimmune disease. This concern is not simply hypothetical – a recent immunotherapy trial demonstrated a close relationship between tumour response, and the development of autoimmune pathology (Gogas *et al.*, 2006). This problem is particularly acute with the use of whole cell immunogens, since all cells share a large number of "common" proteins involved in housekeeping cellular metabolic functions, as well as expressing smaller numbers of "tissue specific" proteins. A striking result from our study was that these "common" proteins do not appear to break tolerance in these experiments, since priming with oxidised ovarian-derived SK-OV-3 and with oxidised melanoma-derived MEL-11 cells generated T cells with minimum cross-reactivity with each others priming antigen. Of course the risk remains of some cross reactivity with normal (untransformed) epithelial tissue, such as breast epithelium, which shares several TAAs, or normal peritoneum. Further studies will be required to define the

exact repertoire of the response, and the potential danger of excess autoimmune reactions.

This study leaves some important questions for future study. The molecular mechanism by which oxidation enhances immunity, and why this can lead to a break in tolerance remains unknown and is the subject of detailed examination in our laboratory. Preliminary results, however, indicate that alterations in Treg activation do not play the major role. Furthermore, it is very likely that the responses generated could be amplified further, for example by blocking negative regulatory interactions between DCs and T cells, such as those mediated by CTLA-4 (Hodi *et al.*, 2003; Ribas *et al.*, 2005) or PD-L1 (Iwai *et al.*, 2002; Nomi *et al.*, 2007). Nevertheless, the results presented here suggest that oxidised tumour cells will provide an excellent and novel starting option as a source of generic antigens for use in DC-based immunotherapy protocols. We are now further optimising antigen loading and DC maturation in ovarian cancer patients with recurrent disease, as the combination of oxidised tumour and DCs could provide a successful adjuvant therapeutic strategy for these patients.

4.5. Conclusions

- DCs derived from ovarian cancer patients that were preloaded with HOCl-oxidised SK-OV-3 were as capable as DCs from healthy volunteers in stimulating ovarian tumour-specific T cell responses.

- DCs derived from ovarian cancer patients were capable of maturation in the presence of agonistic CD40 antibody and MPL. These mature DCs were superior

to immature DCs, and primed stronger $CD4^+$ and $CD8^+$ tumour-specific T cell responses.

- Ovarian cancer patients T cells were not tolerant to autologous DCs pulsed with HOCl-oxidised SK-OV-3, and demonstrated tumour-specificity. These T cells efficiently recognised the immunogens (i.e. SK-OV-3) and also the tumour antigens, HER-2/neu and MUC1 which were overexpressed by SK-OV-3 cells.

- Most importantly, T cells stimulated with DCs loaded with oxidised SK-OV-3 directly recognised autologous ascites (i.e. in the absence of APCs).

- These T cells were tissue and tumour-specific. T cells primed with DCs pulsed with oxidised MEL-11 cells only recognised oxidised MEL-11 and the MART-1 melanoma immunodominant peptide and not the autologous ascites. Similarly, T cells primed with oxidised SK-OV-3 cells recognised oxidised SK-OV-3 but not oxidised MEL-11 cells.

Chapter 5

Optimisation of Anti-Tumour Responses in an Autologous Melanoma Model

5.1. Introduction

In chapter 3, a robust human *in vitro* cell-culture system was developed to examine the use of HOCl-oxidised allogenic ovarian carcicnoma cell line, SK-OV-3, as a potent source of immunogens for monocyte-derived DCs to prime autologous tumour-specific T cells. T cells recognising specific epitopes of HER-2/neu and MUC1 (both being overexpressed on SK-OV-3 cell line and ovarian carcinomas), and to oxidised SK-OV-3 were generated. In chapter 4, the study was further extended in the context of ovarian cancer patients in remission to determine whether these patients' T cells were tolerant to stimulation with autologous DCs preloaded with oxidised SK-OV-3. Strong T cell responses were elicited to HER-2/neu and MUC1, and they were further enhanced with the use of mature DCs. The T cells were also efficient in the direct recognition of autologous ascites, suggesting that oxidised whole cell antigens could be used as potential cancer vaccines. However, this approach could only realistically be tested *in vitro*.

In this chapter, a well-characterised mouse B16.F10 melanoma model was selected for preliminary studies of the immunogenicity of DC pulsed with oxidised tumour cells as cancer vaccines *in vivo*. There are several advantages of this tumour model. First, the B16.F10 melanoma alone are poorly immunogenic due to their low basal expression of MHC Class I, B7-1 and ICAM-1 important for CTL recognition (Casorati *et al.*, 1995; Dezfouli *et al.*, 2003; Itoh *et al.*, 1994; Nishio *et al.*, 1996), and this characteristic makes them ideal for studying HOCl-oxidation and enhancement of tumour immunogenicity. Second, the B16.F10 melanoma overexpresses several defined TAAs, including TRP-1 and 2, gp-100 and MART-1. MHC class I immunodominant epitopes of these TAAs, in particular the TRP-2 (Bloom *et al.*,

1997) have been cloned and well-characterised by several groups, and can be used for evaluating tumour-specific responses after cancer vaccine administration. Third, this tumour model is a good model to explore peripheral tolerance and overcoming such tolerance with DC-based vaccines. The differentiation antigen TRP-2, which is expressed by both normal melanocytes and melanoma for melanin synthesis, is poorly immunogenic (Tang *et al.*, 2007), most probably because of the involvement of peripheral tolerance. Thus a demonstration of the induction of anti-TRP-2 responses by the DC-based vaccine would suggest that peripheral tolerance has been overcome. The results of this autologous tumour mouse model described would provide insights into the parameters that govern successful DC vaccines and would facilitate the optimisation of these vaccines in tumour immunotherapy.

5.2. Objectives

- To determine the TRP-2 expression on B16.F10 melanoma and their sensitivity to HOCl-oxidation and killing.

- To compare and evaluate the different routes of vaccination (i.e. intravenous, intraperitoneal and subcutaneous) with DCs preloaded with oxidised B16.F10 melanoma cells.

- To determine whether HOCl-oxidised B16.F10 cells are superior immunogens for DCs to prime specific T cells than heat-killed or HCl-killed B16.F10 cells.

- To compare and evaluate the immunogenicity of direct immunisation of mice with oxidised B16.F10 melanoma cells in the absence of DCs to immunisation with DCs preloaded with oxidised B16.F10 cells.

5.3. Results

5.3.1. B16.F10 melanoma overexpress tyrosinase-related protein 2

The expression of TRP-2 was previously reported (Bloom *et al.*, 1997; Tsukamoto *et al.*, 1992) and confirmed by western blot. Antibodies to TRP-2 identified a single band with molecular weight of approximately 75 kDa (Fig. 5.1). Its expression was absent in the CHO cell line.

5.3.2. HOCl induces necrosis in B16.F10 melanoma

The susceptibility of B16.F10 cells to HOCl-oxidation killing was measured by PI staining and shown in Fig. 5.2. Treatment of B16.F10 cells with increasing concentrations of HOCl resulted in a dose dependent increase in the % of unpermeabilised cells which took up PI (gate M1, Fig. 5.2A, quantified in Fig. 5.2B). Viable cells are impermeable to PI, and the % PI positive cells gave a measure of dead cells. More than 99% of cell death was consistently observed at 60 μM HOCl and above, and this concentration was selected for use in all further experiments. Cellular necrosis was also determined by measuring the DNA content with PI staining. It clearly showed the presence of the G_1 and G_2 peaks and no sub-G_0 staining which corresponded to fragmented DNA from cells undergoing apoptosis.

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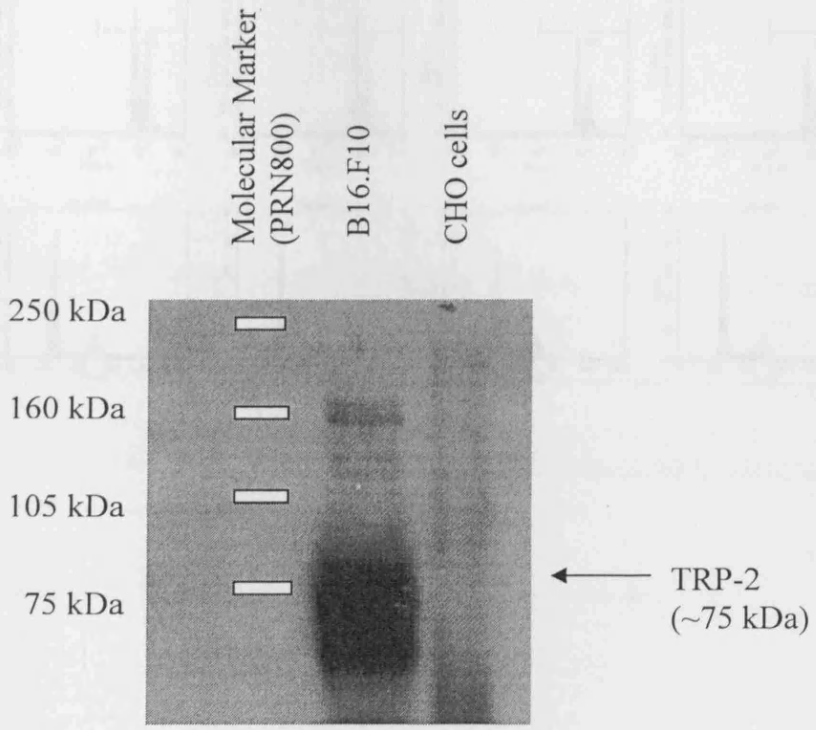
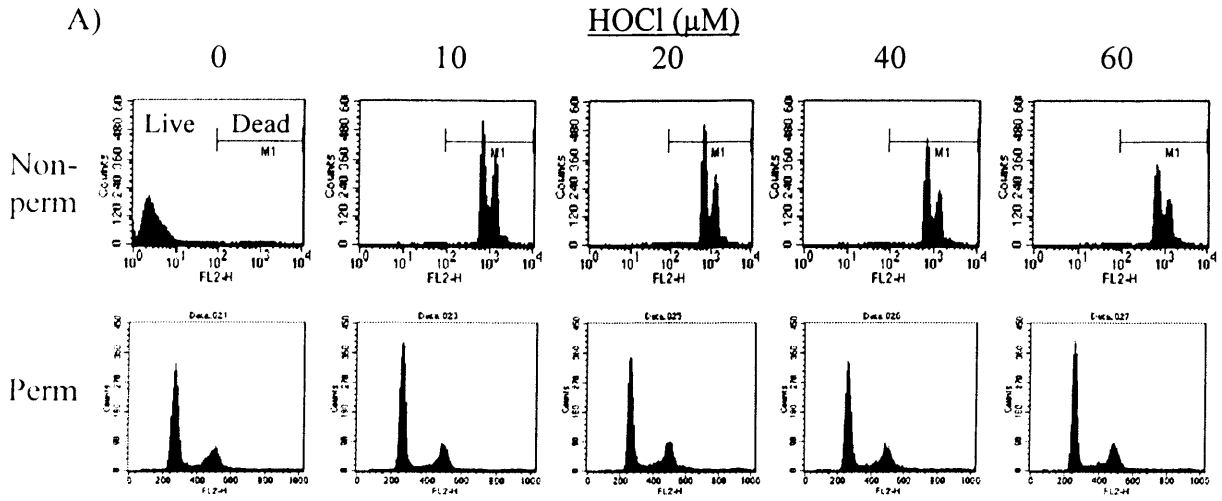


Figure 5.1 Expression of TRP-2 tumour antigens on B16.F10 melanoma.1 x 10^6 B16.F10 melanoma or CHO cells were lysed in sample buffer, resolved on 12.5% SDS-PAGE electrophoresis, and transferred to nitrocellulose membranes as described in Materials and Methods. TRP-2 expression was detected by the polyclonal anti-PEP8 antibody that specifically recognises TRP-2 in melanocytes. It was detected as a single band of approximately 75 kDa. CHO cells were negative for TRP-2 expression. Data are representative of 3 independent experiments.



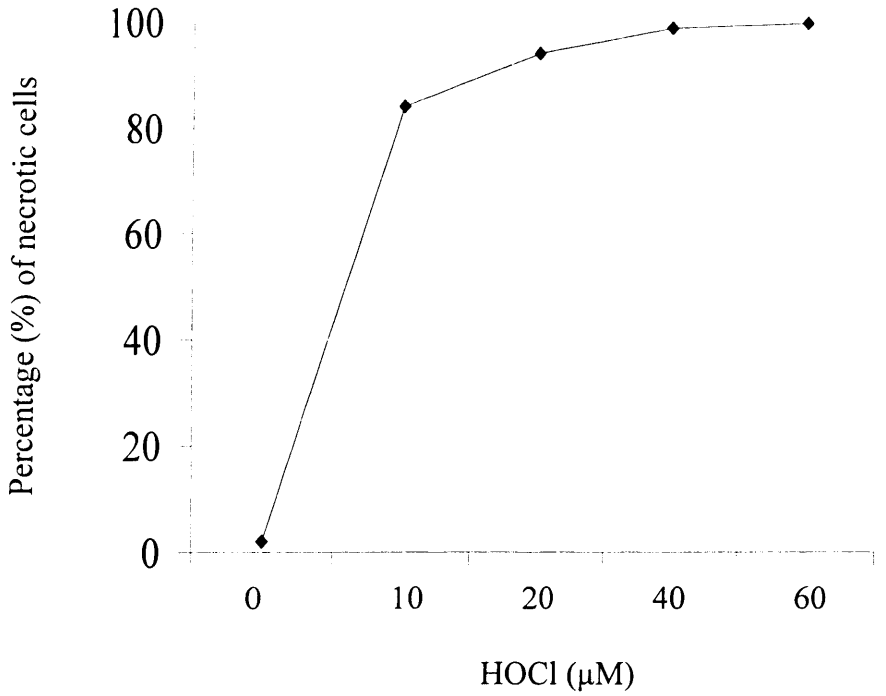


Figure 5.2 Dose dependent necrosis of B16.F10 melanoma treated with HOCl. A and B) B16.F10 melanoma cells were incubated with different concentrations of HOCl as indicated for 1 h at 37°C. Cells were washed twice and stained with PI as described in Materials and Methods either without (non-perm, A top row) or after permeabilisation with 70% ethanol (perm, A bottom row). The percentage of highly PI positive cells (M1 gate) corresponded to percentage of dead cells was plotted against HOCl concentration in B. Data are representative of 5 independent experiments.

5.3.3. Phenotypic profile of mouse bone marrow-derived dendritic cells

To evaluate the CD80, CD86 and MHC Class II expression on immature bone marrow-derived DCs, and to determine whether they could respond and mature in the presence of LPS and MPL, the DCs were treated with low dose (100 ng/ml) or high dose (1000 ng/ml) of the maturation stimuli. IFN-γ is a strong stimulator of marcophages and DCs, and has been shown to induce upregulation of maturation markers on DCs (He *et al.*, 2007). IFN signaling is controlled via the promoters of IFN-inducible genes (Pestka *et al.*, 2004), and it has been demonstrated recently that the interferon-regulatory factor (IRF)-8/IFN consensus sequence-binding protein is involved in TLR signaling and contributes to the cross-talk between TLR and IFN-γ signaling pathways (Scharton-Kersten *et al.*, 1997). Hence DC maturation could be augmented in the presence of TLR agonist(s) stimulation. For this purpose, rat recombinant IFN-γ (100 U/ml) was added at the same time as the stimuli and incubated for 24 h at 37°C, 5% CO_2. Fig. 5.3A showed the typical dot-plot profiles of isotype control, PE-labelled-CD11c DCs, and FITC-labelled MHC Class II DCs. In Fig. 5.3B, representative fluorescence histograms showed upregulation of DC maturation markers and costimulatory molecules – CD80, ICAM and MHC Class II – in both low and high doses of LPS or MPL plus IFN-γ. The median fluorescence values and the % of DCs in the M1 gate are shown in the top right corner of the histograms. DCs treated with IFN-γ showed a small upregulation of these maturation markers, while the expression of these markers was low on untreated DCs. The CD80 and ICAM expression were further enhanced, although slightly, when treated with LPS or MPL (both low and high doses) in addition to IFN-γ. Higher percentage of these DCs compared to that of untreated DCs also appeared in the M1 gate as showed in the figure. For MHC Class II expression, it was upregulated in the presence of LPS

or MPL plus IFN-γ when compared to that of untreated DCs. However, the use of IFN-γ in addition to LPS or MPL did not cause further upregulations on the DCs compared to IFN-γ treatment alone. Surprisingly, the level of CD80, ICAM and MHC Class II expression on these LPS/ MPL and IFN-γ treated DCs were low compared to those reported in the literature (Escors *et al.*, 2008; Veeraswamy *et al.*, 2003; Winzler *et al.*, 1997). One highly possible reason was that the LPS and MPL that were used in this experiment had lost their usual potency. Another possible reason might be that a longer LPS, MPL and IFN-γ treatment time (e.g. 48 h instead of 24 h used here) and/or a high dose of the stimulus was required. Hence further optimistion of this maturation protocol is necessary. The CD86 expression on these treated DCs was not shown here because its level of upregulation was very small even after treatment with LPS or MPL plus IFN-γ, in comparison to untreated DCs and also to markers such as CD80, ICAM and MHC Class II. This effect has also been demonstrated by other groups (M. Collins, personal communications).

5.3.4. *In vivo* administration of DCs loaded with HOCl-oxidised B16.F10 tumour cells primed anti-tumour responses in C57BL/6 mice

To evaluate the immunogenicity of bone marrow-derived DCs loaded with oxidised B16.F10 cells as cancer vaccines, 6-8 weeks old C57BL/6 mice were immunised via the tail vein with day 6 DCs loaded with the oxidised tumour cells (each mouse received 50 µl of HBSS containing 1 x 10^6 DCs that had been preloaded with 1 x 10^6 oxidised B16.F10). 60 µM HOCl was selected for treating the tumour cells because it consistently induced cellular necrosis in more than 99% of the B16.F10 cells. Also in chapters 3 and 4, this concentration had been used in the human *in vitro* studies and found to induce robust anti-tumour responses. Two sets of negative controls were set

cultured in complete IMDM media but no tumour cells. In preliminary studies, we isolated the spleen cells from the mice after two weeks of *in vivo* priming (i.e. vaccination) and assessed their responses directly in an IFN-γ ELISPOT assay. However, high background responses were seen with the spleen cells that were taken from the mice immunised with DCs loaded with oxidised B16.F10 cells or TRP-2 peptides, and subsequently cultured with complete RPMI media only in the ELISPOT. A possible reason for this high background could be due to the presence of residual oxidised B16.F10 cells on the APCs. Such APCs in the spleen cell population could present the residual antigens to specific T cells, leading to high IFN-γ production without the need of adding exogenous antigens in the assay. No such high background responses were seen in the ELISPOT with spleen cells taken from mice immunised with DCs and no tumour cells or with HBSS alone. To increase specificity, the spleen cells were cultured *in vitro* for a further week, either in the presence of additional oxidised B16.F10 or with complete RPMI media only. Viable cells were then collected, and tested in the IFN-γ ELISPOT for their responses to oxidised B16.F10 cells, TRP-2 H-2K^b restricted peptide, or complete RPMI. The general protocol is showed in Fig. 5.4.

In Fig. 5.5A (black bars), spleen cells from mice immunised with DCs pulsed with oxidised B16.F10 exhibited strong responses to the oxidised B16.F10 (>180 spots per million spleen cells) and TRP-2 peptides (>150 spots per million spleen cells) in the ELISpot assay after 1 week of *in vitro* antigen restimulation and expansion. This suggested that antigen-specific T cells priming by the DC-oxidised B16.F10 priming had occurred *in vivo*. Furthermore the high background IFN-γ secretion by these spleen cells as previously mentioned was not seen when they were restimulated and

expanded for a further week with RPMI media alone (white bars; <50 spots per million spleen cells). This suggested that antigen-specific T cells in the spleen population died due to the lack of appropriate stimulation by presenting APCs. In this same population of antigen-stimulated T cells, high IFN-γ secretion was seen in the media alone group in ELISPOT. This high IFN-γ secretion observed in the former could be explained by the presence of APCs that were being kept alive by the 1 week antigen stimulation and presenting residual oxidised B16.F10 cells on their cell surface to antigen-specific T cells, leading to high IFN-γ production in the assay.

It was noted that the tumour-specific responses in the ELISPOT for this mouse melanoma model were considerably lower (about 10-fold lower) to those obtained in the studies involving healthy volunteers and ovarian cancer patients. Though oxidised tumour cells were used in these two studies, they were different cell lines obtained from two different species and thus difficult to directly compare. However, the anti-tumour responses might be improved by increasing the ratio of tumour cells to DCs (e.g. four tumour cells to one DC) to maximise the chance of antigen uptake by the DCs. Very low responses were observed from the spleen cells of mice immunised with either DC pulsed with complete IMDM media (Fig. 5.5B, black bars) or with HBSS only (Fig. 5.5C, black bars). Similar poor results were obtained when the spleen cells were restimulated with RPMI media alone in all the groups (Fig. 5.5B, C; white bars). To determine the precursor frequency of melanoma-specific T cells in the naïve spleen of C57BL/6 mice, spleen cells were isolated and directly tested in the ELISPOT for their IFN-γ production (Fig. 5.6). No IFN-γ responses were detected to oxidised B16.F10, TRP-2 peptides or to the media.

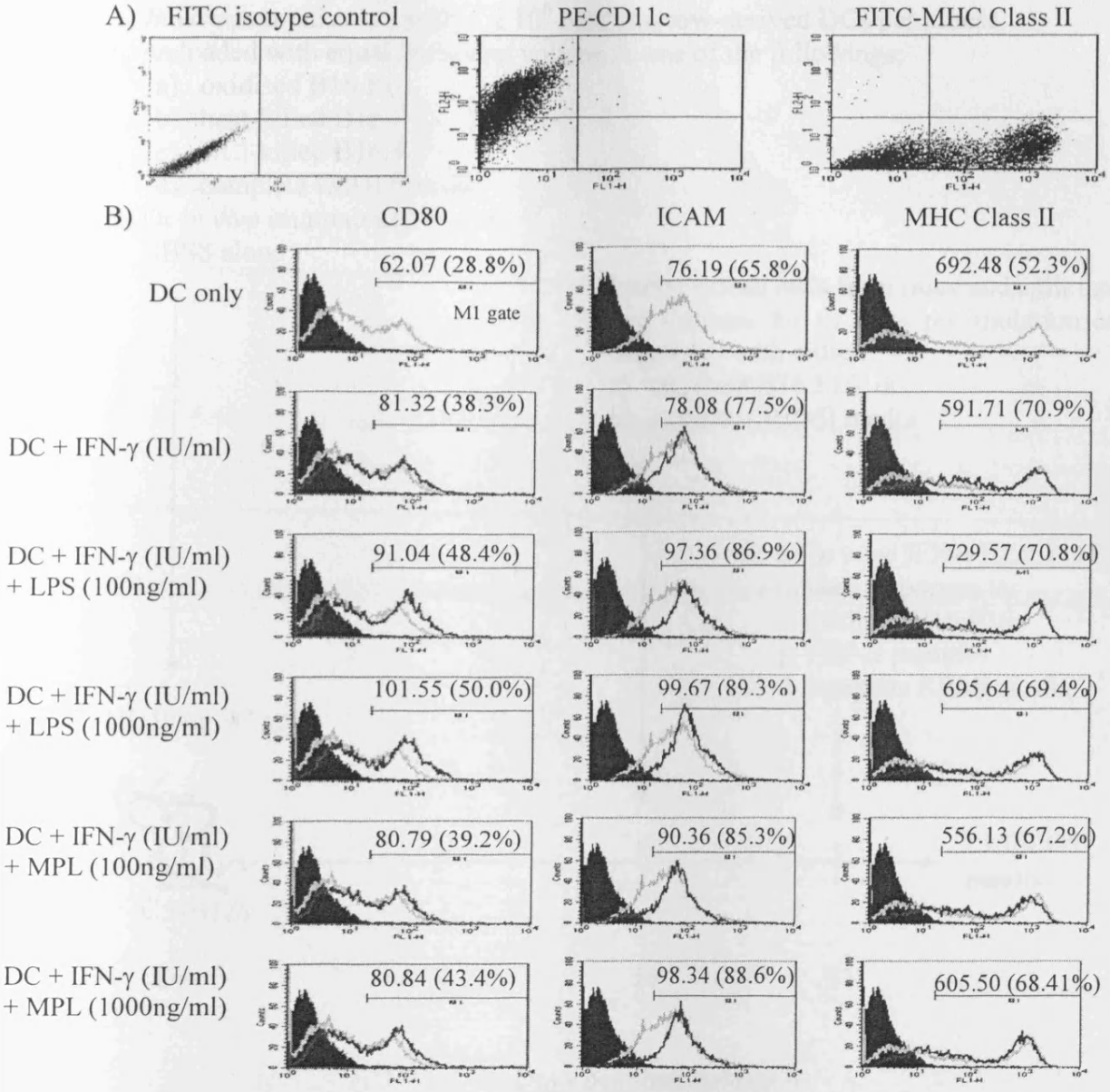


Figure 5.3 Bone marrow-derived DCs matured in the presence of LPS, MPL and IFN-γ. Day 6 bone marrow-derived DCs were treated for 24 h with IFN-γ (100 IU/ml) together with a low dose LPS/MPL (100ng/ml), or high dose LPS/MPL (1000 ng/ml). For negative control, DCs were treated with complete IMDM media alone (i.e. untreated DCs). DC cultures were also set up to evaluate the effect of IFN-γ alone. After treatment, DCs were double-stained with PE-conjugated CD11c and FITC-conjugated antibodies to CD80, ICAM or MHC Class II as described in Materials and Methods. A) Representative dot-plots of CD11c, MHC Class II DCs and FITC-isotype control antibodies staining. B) One representative frequency histogram of fluorescence (FL1 channel) in the absence of stimulus, or in the presence LPS or MPL plus IFN-γ, or IFN-γ alone. The number in the right hand top corner of each histogram shows the MFI of the total population (channel number), while the figures in brackets showed the % of cells in the M1 gate. The black filled profile shows negative control staining with control FITC-conjugated IgG, the black line profile shows different treatement, while the grey line profile shows untreated DCs for comparison.

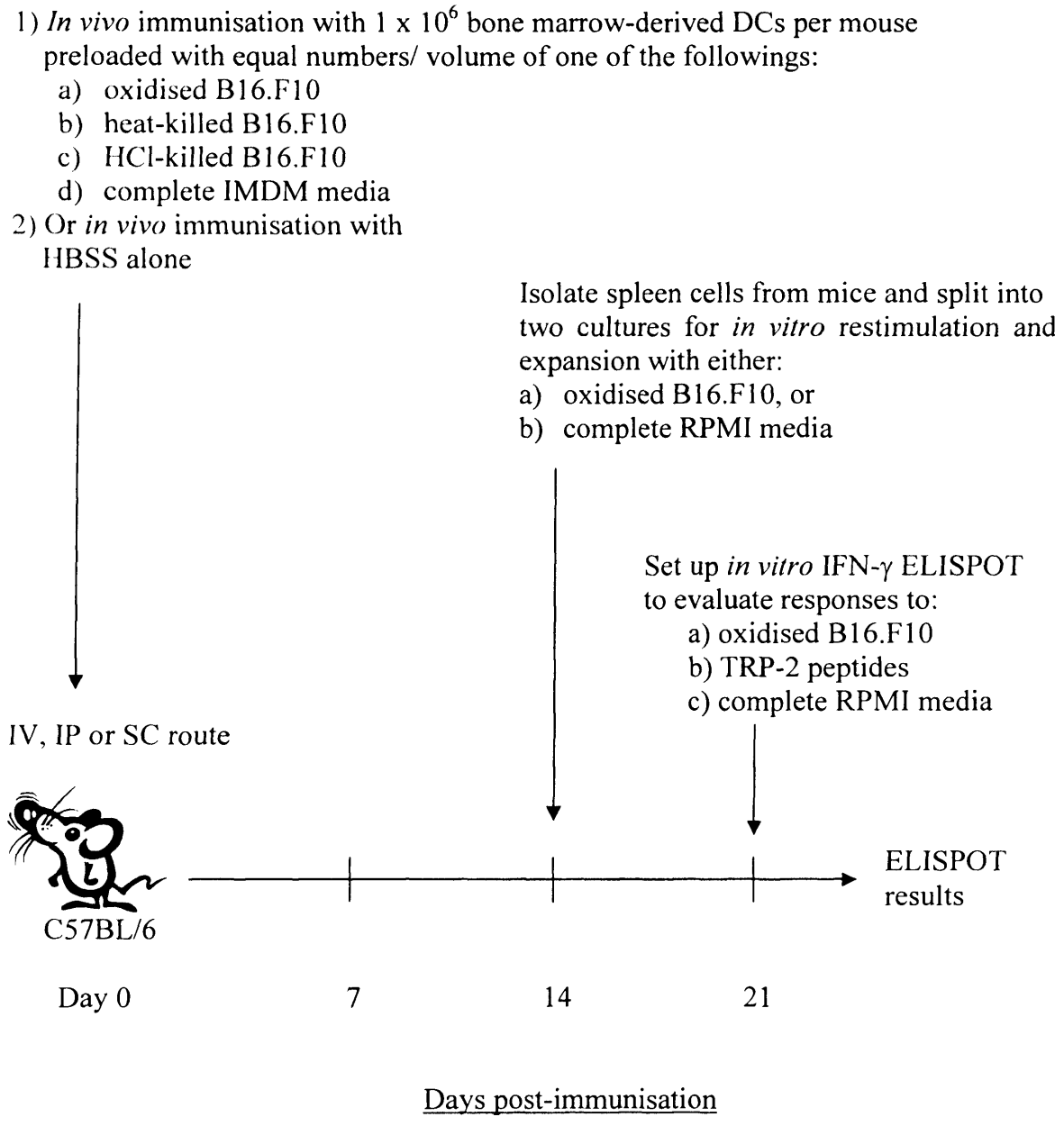


Figure 5.4 A schematic diagram showing the experimental approach of *in vivo* administration of bone marrow-derived DCs pulsed with oxidised B16.F10 to C57BL/6 mice and evaluation of their IFN-γ production in ELISPOT following 1 week of restimulation in the presence or absence of oxidised B16.F10. Mice were immunised via the intravenous, intraperitoneal or subcutaneous route in the tail vein with 1 x 10^6 DCs pulsed with equal number of oxidised B16.F10 (ratio of DCs to tumour cells was 1:1) per mouse. Two weeks later, the spleens were isolated and divided into 2 cultures; one was restimulated with oxidised B16.F10 (ratio of 1 tumour cell to 10 spleen cells) and the other restimulated with complete RPMI without antigen. IFN-γ production was measured by testing the primed spleen cells (1 x 10^6 cells/well) responses to fresh APCs (1 x 10^5 cells/well) with oxidised B16.F10 (1 x 10^5 cells/well), TRP-2 peptides (final of 1 μM) or in complete RMPI only in the ELISPOT.

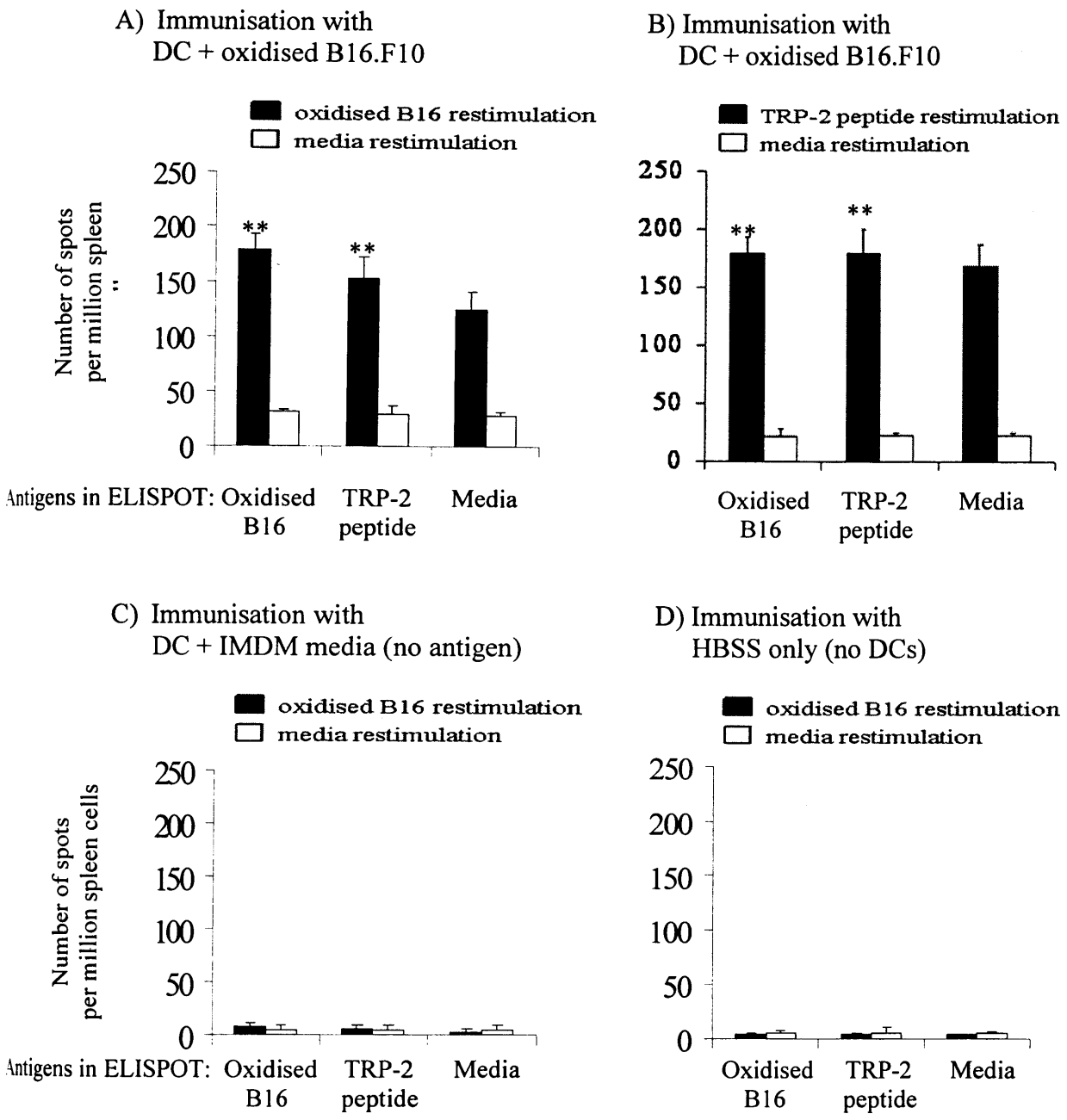


Figure 5.5 *In vivo* administration of bone marrow-derived DCs pulsed with oxidised B16.F10 primed a potent anti-tumour response in C57BL/6 mice. Mice were immunised in the tail vein with A and B) $1x10^6$ DCs pulsed with equal number of oxidised B16.F10 (ratio = 1:1) per mouse, C) $1x10^6$ DCs pulsed with complete IMDM media, or D) HBSS only. After 2 weeks, the spleens were isolated and divided into 2 cultures: restimulated with antigens (i.e, oxidised B16.F10 or TRP-2 peptides) or with complete RPMI only. IFN-γ production was measured by ELISPOT. The results are the means ± standard error of the mean of at least six independent experiments (i.e. spleen cells from at least 6 different mice) per group. The asterisk indicates those columns differing significantly (**, $P<0.001$, paired Student t test) from the RPMI media control. The background secretion of IFN-γ by spleen cells was low in all the groups when they were restimulated with RPMI media alone (white bars).

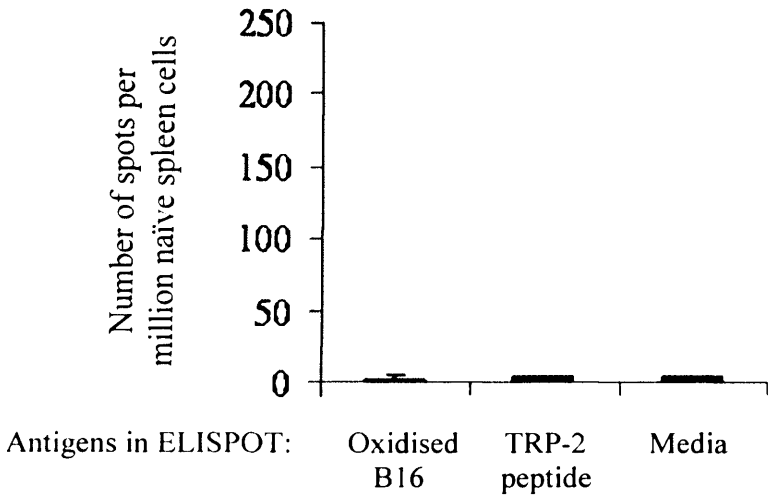


Figure 5.6 Naïve spleen cells from C57BL/6 mice did not response to oxidised B16.F10 or to TRP-2 peptides. Naïve spleen cells were isolated from C57BL/6 mice (i.e. without prior administration with DCs pulsed with oxidised B16.F10 cells or with TRP-2 peptides), and directly tested in the ELISPOT for their IFN-γ production. The results are the means ± standard error of the mean of three independent experiments (i.e. spleen cells from 3 different mice).

5.3.5. Intravenous route of administration of DC preloaded with oxidised B16 is superior to intraperitoneal and subcubtaneous routes for the induction of tumour-specific responses

DC presumably must home to secondary lymphoid organs to prime T cell responses, and the route of administration of such antigen-loaded DCs *in vivo* might significantly affect the overall success of the vaccines. Several studies showed that differential immune responses are induced using different routes of immunisation in both mice and humans (Eggert *et al.*, 1999; Fong *et al.*, 2001). In the case of mice, subcutaneous and intraperitoneal immunisations but not intravenous immunisation had primed potent anti-tumour responses that led to B16 tumour rejection *in vivo* (Eggert *et al.*, 1999). To determine the best route of immunisation of mice with DCs preloaded with oxidised B16.F10, we immunised the mice with antigen-loaded DCs via three different routes, intravenous (IV), subcutaneous (SC) or intraperitoneal (IP) and evaluated spleen cells anti-tumour responses in the IFN-γ ELISPOT after 2 weeks of vaccination as detailed in Materials and Methods.

As shown in Fig. 5.7, the IV route of DC vaccination was the most potent in inducing tumour-specific T cells that recognised the oxidised B16.F10 and TRP-2 derived peptides (Figs. 5.7A and B first columns, •) as compared to SC or IP routes after a further week of restimulation and expansion with oxidised B16.F10. An average of 175 spots and 150 spots per million spleen cells responding to the oxidised B16.F10 and TRP-2 peptides, respectively, were observed with the IV route. Contrary to the findings by other groups, the SC route of vaccination gave the poorest anti-tumour responses (an average of 50 spots per million spleen cells observed for oxidised B16.F10, and 75 spots per million per spleen cells recognising TRP-2 peptides) [Figs.

5.7A and B third columns, •]. It was noted that results obtained from the SC route of vaccination were difficult to replicate and inconsistent. Although the IP route induced responses to oxidised B16 and TRP-2 peptides (Figs. 5.7A and B middle columns, •), the magnitude of the responses was smaller compared to that of I.V route. Very low responses (approximately 35 spots per million spleen cells) were seen from spleen cells restimulated with media only across all vaccination routes (Fig. 5.7, ■).

5.3.6. Oxidised B16.F10 and not heat-killed or hydrochloric acid killed B16.F10 are potent immunogens for priming anti-melanoma responses

In previous study, it was showed that HOCl-oxidised tumour cells had enhanced immunogenicity and were more potent as a source of antigen for human DCs to prime anti-tumour T cells. It was found that the enhanced immune response seen was due specifically to oxidation by HOCl and not simply a result of cell necrosis (Fig. 3.4). In this current study, necrosis in B16.F10 cells was induced via HOCl oxidation, a brief exposure to acid (1 M HCl, 60 sec) or with heat treatment (56°C, 30 min). Under these conditions, the B16.F10 cells retained cellular integrity but were >99% dead by necrosis (confirmed by trypan blue and PI staining, data not shown). Then we loaded the DCs with one of these necrotic B16.F10 cells and immunised the mice via the IV route (it was selected as it gave the best anti-tumour responses compared to SC and IP routes as determined previously). The results confirmed our previous findings; the spleen cells that were primed using DC loaded with oxidised B16.F10 gave strong tumour-specific responses (Fig. 5.8A) whilst DC loaded with either acid-killed (Fig. 5.8B) or heat-killed (Fig. 5.8C) tumour cells *in vivo* failed to prime a significant immune response to any antigen tested in the IFN-γ ELISPOT. It was again noted that the population of antigen-stimulated T cells exhibited high IFN-γ secretion in the

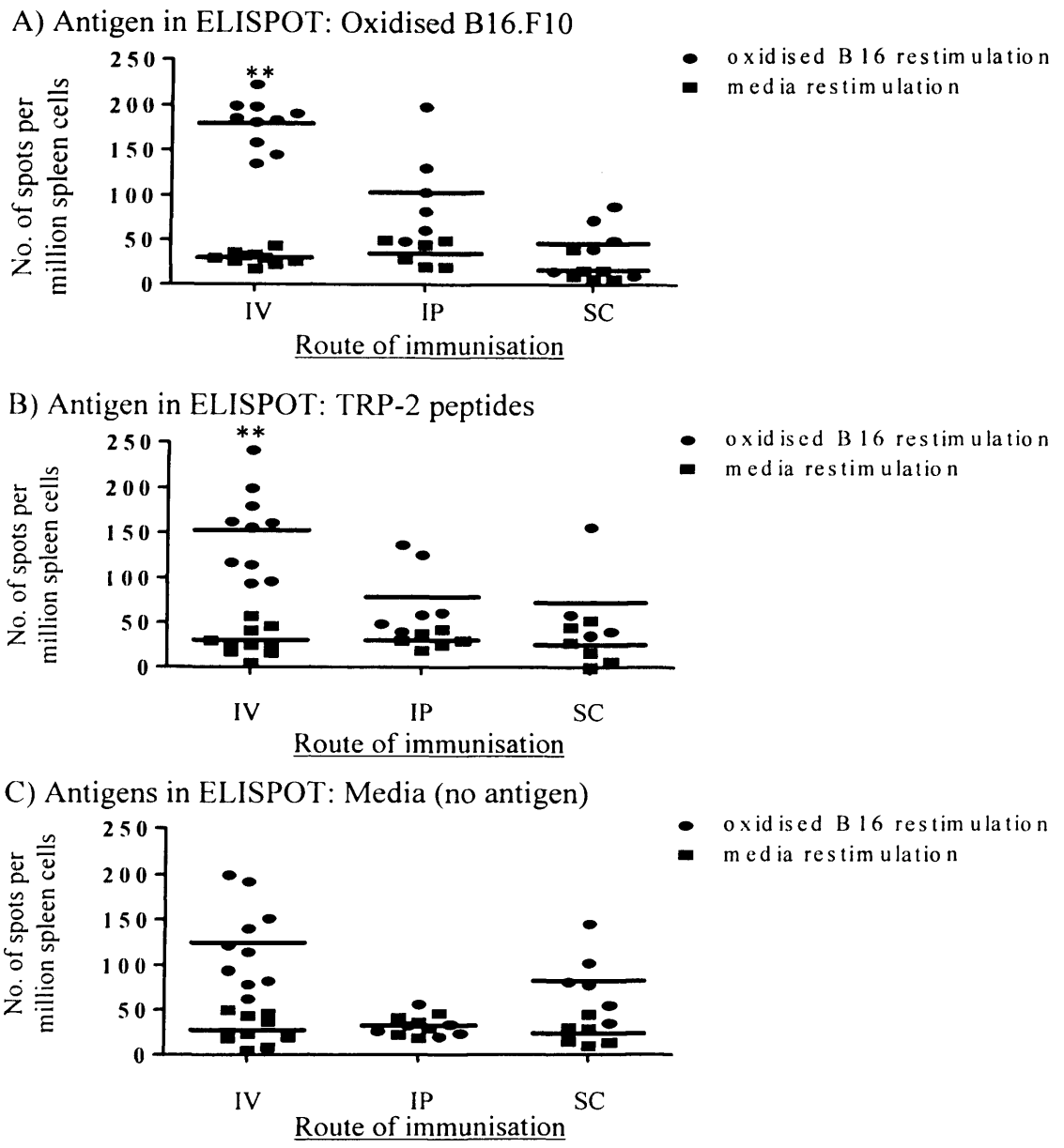


Figure 5.7 Intravenous administration of DCs loaded with oxidised B16.F10 primed a more potent tumour-specific response than immunisation via the intraperitoneal or subcutaneous route. Mice were immunised intraveously (IV) in the tail vein, intraperitoneally (IP) in the right peritoneum, or subcutaneously (SC) at the right flank with 1 x 10^6 DCs pulsed with equal number of oxidised B16.F10 (ratio = 1:1) per mouse. After 2 weeks, spleen cells were isolated and divided into 2 cultures; one was restimulated with oxidised B16.F10 and the other with complete RPMI. IFN-γ production was measured by ELISPOT. Each point represents the average of triplicate cultures of a different mouse. The line shows the median response. The responses of spleen cells restimulated with oxidised B16.F10 (●) is significantly greater than the RPMI media control (■) in the same IV group (**, $P < 0.01$; Mann-Whitney). The responses of the spleen cells restimulated with oxidised B16.F10 (●, first columns) in the IV group were also significantly greater than that of spleen cells restimulated with oxidised B16.F10 in the IP (●, second columns) and SC groups (●, first columns) [**, $P < 0.01$; Mann-Whitney]. Background IFN-γ secretion of spleen cells restimulated with RPMI media alone was low (■).

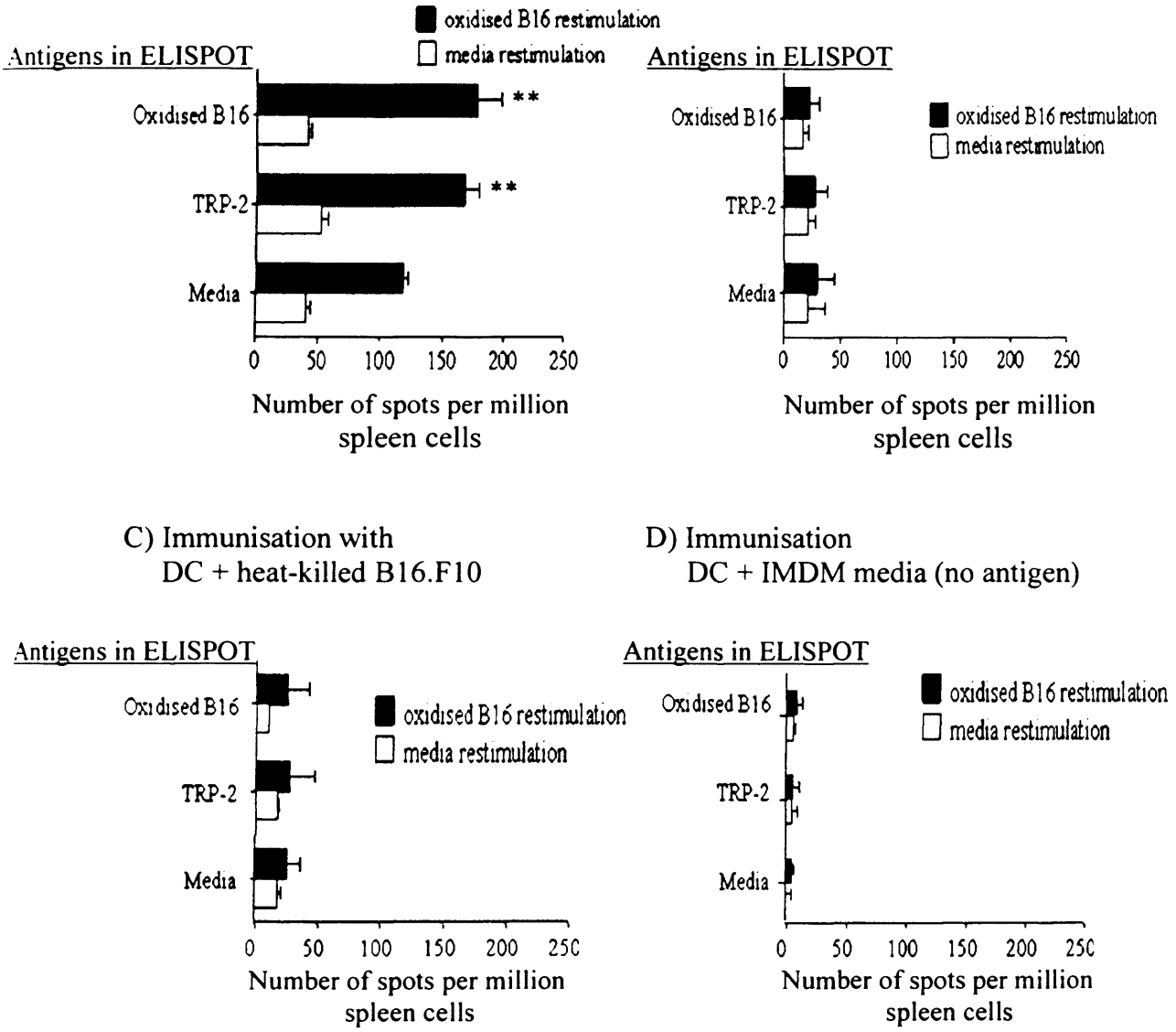
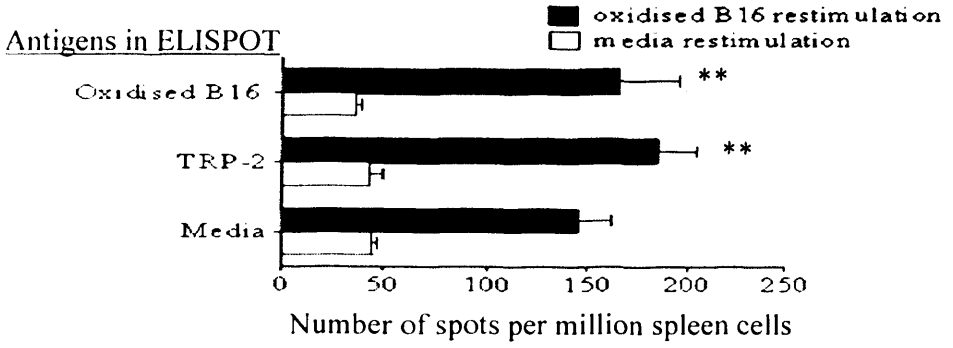
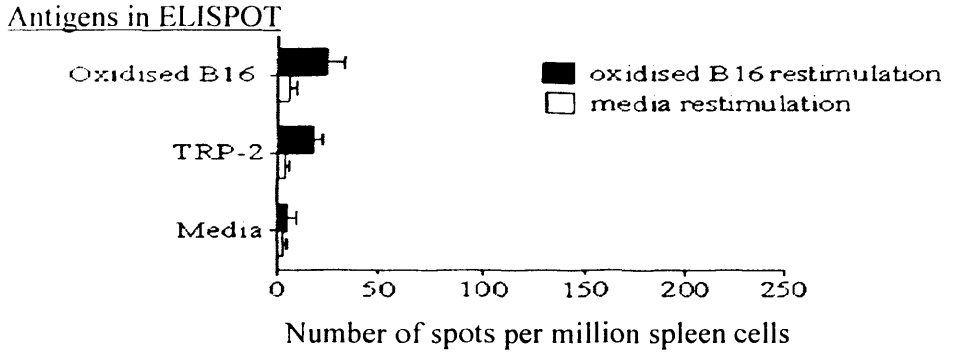


Figure 5.8 DCs loaded with HOCl-oxidised B16.F10 cells, but not heat-killed or HCl-killed B16.F10, or IMDM media stimulated T cells specific to both B16.F10 melanoma cells and epitope of TRP-2. Mice were immunised via the IV route in the tail vein with DCs pulsed with B16.F10 killed with A) HOCl-oxidation, B) heat, or C) HCl (ratio of DC to tumour cell = 1:1). The spleens were isolated 2 weeks later and divided into 2 cultures; one was restimulated with HOCl-oxidised B16.F10 and the other restimulated with complete RPMI without antigen. IFN-γ production was measured by ELISPOT. The results are the means ± standard error of the mean for at least three independent experiments (i.e. spleen cells from three different mice). The asterisk indicates those columns differing significantly (**, $P<0.001$) from the RPMI media control (paired Student *t* test). The background secretion of IFN-γ by spleen cells was low in all the groups when they were restimulated with RPMI media alone (white bars).

A) Immunisation with DC loaded with oxidised B16.F10



B) Immunisation with oxidised B16.F10 only



C) Immunisation with DC pulsed with IMDM media (no antigens)

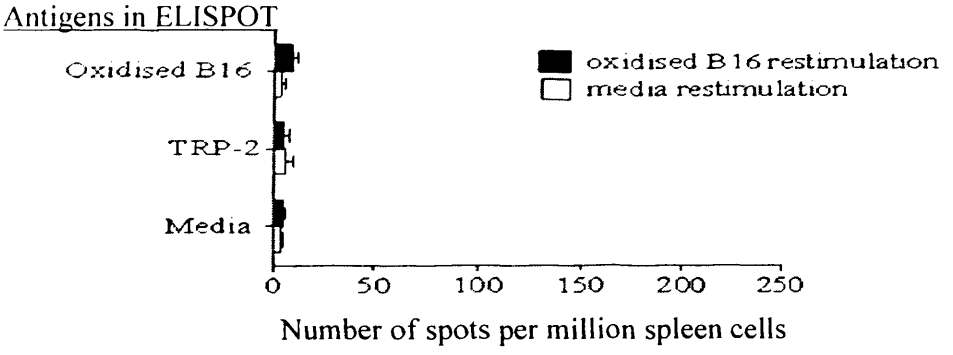


Figure 5.9 DCs loaded with oxidised B16.F10, but not oxidised B16.F10 in the absence of DCs or DC alone stimulated T cells specific to both B16.F10 cells and epitope of TRP-2. Mice were immunised via the IV route in the tail vein with A) DCs pulsed with oxidised B16.F10, B) oxidised B16.F10 alone, or C) DC pulsed with IMDM media. The spleens were isolated 2 weeks later and divided into 2 cultures; one was restimulated with oxidised B16.F10 and the other with complete RPMI without antigen. IFN-γ production of the spleen cells was measured by ELISPOT. The results are the means ± standard error of the mean for at least four independent experiments (i.e. spleen cells from four different mice). The asterisk indicates those columns differing significantly (**, $P<0.001$) from the RPMI media controls (paired Student *t* test). The background secretion of IFN-γ by spleen cells was low in all the groups when they were restimulated with RPMI media alone (white bars).

media alone group in ELISPOT. This could again be explained by the presence of APCs that were being kept alive by the 1 week antigen stimulation and presenting residual oxidised B16.F10 cells on their cell surface to antigen-specific T cells, casuing high IFN-γ production in the ELISPOT.

5.3.7. DC is required for the *in vivo* processing and presentation of B16 oxidised tumour cells *in vivo*

A further analysis was carried out to investigate whether DCs were required for the processing and presentation of oxidised B16.F10 *in vivo*, or the oxidised B16.F10 could be taken up by the migrating APCs in the blood stream and be processed and presented to T cells, or accumulated in the lymphoid organs as a source of antigens for APCs. As it had been determined previously that IV route of immunisation gave the best anti-tumour responses, it was selected as the route of immunisation in this set of experiments. The mice were immunised via the IV route with DCs preloaded with oxidised B16.F10 or with oxidised B16.F10 only (i.e. no DCs) for 2 weeks, then their spleen cells were isolated and restimulated with oxidised B16.F10 or with RPMI media only. As a negative control, some mice were immunised with DCs that were pulsed with IMDM media only. Strong anti-tumour responses were observed in mice immunised with DCs preloaded with oxidised B16.F10 (Fig. 5.9A, black bars) and not with oxidised B16.F10 alone (Fig. 5.9B, black bars) or with DCs plused with complete IMDM media (Fig. 5.9C, black bars). Low background responses were seemed across all groups when the spleen cells were restimulated with RPMI media only (Fig. 5.9, white bars). This confirmed that DCs were required for the processing and presentation of oxidised B16.F10 *in vivo*. The absence of anti-tumour immunity observed with oxidised B16.F10 only immunisation might be that the oxidised tumour

cells were rapidly cleared from bloodstream after IV injection before reaching the lymphoid organs or other APCs.

5.4. Discussion

Results presented in chapters 3 and 4 strongly suggested that oxidised SK-OV-3 ovarian tumour cells were potent immunogens for DCs in priming anti-tumour T cell responses, and could be used as a therapeutic approach in treating ovarian cancer patients in remission. The well-characterised B16.F10 melanoma C57BL/6 mouse model was being selected for the preliminary study of the immunogenicity of this DC-based immunotherapy *in vivo*. This tumour mouse model has been widely studied by several groups. The B16.F10 melanoma overexpresses the TRP-2 tumour antigen of which a MHC Class I epitope has been identified, and could help to facilitate the analysis of anti-tumour responses following *in vivo* priming.

To establish the potential of B16.F10 killed with HOCl-oxidation as immunogens for DCs, C57BL/6 mice were each immunised via the tail vein with 1 x 10^6 bone-marrow derived DCs previously loaded with 1 x 10^6 HOCl-oxidised B16.F10 cells. It was observed that the DC vaccines were well-tolerated and no toxicity was observed in the mice immediately or 2 weeks after vaccination. It was also noted that no obvious abnormality was seen in the immunised animals, as dissection of the animals 2 weeks after vaccination to isolate the spleen cells for restimulation revealed perfectly normal spleen and other internal organs. There was also no change or depigmentation of their coat-color, which is a sign of autoimmunity. However a detailed analysis of autoimmunity, such as measuring auto-antibodies, would be required to rule out this possibility. In initial IFN-γ ELISPOT assays, the spleen cells from the immnunised

mice were evaluated without a further week of restimulation with antigens (i.e. oxidised B16.F10 or TRP-2) or complete RPMI media only. High background responses were seen in the spleen cells in the absence of antigens (i.e. media only) in the ELISPOT. This might be due to APCs presenting residual oxidised B16.F10 cells to antigen-specific T cells in the spleen cell population. To increase the assay specificity, the spleen cells were divided into 2 cultures with one being restimulated with oxidised B16.F10 cells or TRP-2, and the other with complete RPMI media without antigen. After 1 week of *in vitro* restimulation and expansion, the spleen cells from mice immunised with DCs preloaded with oxidised B16.F10 developed immune responses to the TRP-2 derived peptides and to the oxidised B16.F10 (Fig. 5.5A). No responses were seen in the spleen cells of the same animal when they were restimulated with complete RPMI media alone as the antigen-specific T cells died in culture without receiving appropriate antigen stimulation.

To further confirm that the responses were indeed specific to B16.F10 and to TRP-2 which was highly expressed on these cells, parallel experiments were set up to immunise mice with the DC vaccines. After 2 weeks, the spleen cells were isolated and restimulated with TRP-2 peptide or with complete RPMI media only (Fig. 5.5B). Anti-tumour responses were seen in spleen cells restimulated with TRP-2 peptide and not with media alone. These results were being further supported when no IFN-γ production was seen in mice immunised with HBSS only or DC pulsed with IMDM media (Fig. 5.5C, D). In addition, naïve spleen cells from C57BL/6 mice were isolated and tested in IFN-γ ELISPOT and found not to response to either oxidised B16.F10 or TRP-2 peptides (Fig. 5.6).

In line with the results first shown in chapter 3, the TRP-2 epitope was being cross-presented by the DCs to T cells. It was observed that the tumour-specific responses in the ELISPOT in the C57BL/6 melanoma model were considerably lower (about 10-fold lower) to those obtained in the studies involving healthy volunteers and ovarian cancer patients. The results from these studies were difficult for direct comparison as two very different tumour cell lines derived from two different species were used. However, the anti-tumour responses in this mouse melanoma model might be improved by increasing the ratio of tumour cells to DCs (e.g. four tumour cells to one DC) to maximise the chance of antigen uptake by the DCs and enhancing their antigen presentation capacity by stimulation with DC maturation stimuli such as MPL or CpG. Other B16.F10 derived TAA peptides such as TRP-1 and MAGE would be useful in the IFN-γ ELISPOT analysis to define the breadth of the anti-tumour responses. The results nevertheless established the potential use of oxidised B16.F10 in DC-based immunotherapy of melanoma in C57BL/6 mice.

It has been shown that the route of administration of antigen-loaded DCs *in vivo* might significantly affect the overall success of the vaccines, and differential immune responses were being induced via different routes of immunisation in both mice and humans (Eggert *et al.*, 1999; Fong *et al.*, 2001). One study clearly demonstrated that immunisation via the subcutaneous and intraperitoneal routes and not intravenous route primed potent anti-tumour responses that led to B16 tumour rejection *in vivo* (Eggert *et al.*, 1999). It showed that the TRP-2 peptide-loaded DCs injected subcutaneously accumulated preferentially in the lymph nodes and to lesser extend in the spleen. In the same study, similar observation was observed with intraperitoneal immunisation. However, this study demonstrated that the intravenous route of

immunisation was superior to that of subcutaneous and intraperitoneal. One reason could be that the different antigen used (i.e. whole oxidised B16.F10 versus TRP-2 peptide in the other study) might influence the DCs processing and presentation of the antigen, and the subsequent activation of the T cells. The dose of the antigens and variation of the vaccination protocols, which are both very difficult to compare, could also affect the outcome of the immune response. In chapter 3, HOCl-oxidised tumour cells and not tumour cells made necrotic by heat or HCl were able to prime therapeutic anti-tumour responses *in vitro*. This was further validated *in vivo*. C57BL/6 mice were immunised with DC pulsed with one of the following: oxidised B16.F10, heat-killed B16.F10 or HCl-killed B16.F10. Spleen cells were isolated after 2 weeks of *in vivo* priming and restimulated in culture with the relevant antigen (i.e. oxidised, heat-killed or HCl-killed B16.F10) or with complete RPMI media only. Strong immune responses were observed in the spleen cells of mice receiving DCs pulsed with oxidised B16.F10 (Fig. 5.8). This therefore confirmed that oxidised B16.F10 cells, and not heat-killed or HCl-killed B16.F10 cells, were potent immunogens for DCs. The mechanism of HOCl enhancement was not pursued in this study. However, it was showed that oxidised but not live SK-OV-3 ovarian carcinoma cells were readily taken up by human monocyte-derived DCs (Fig. 3.3). It could be hypothesised that HOCl-treatment of tumour cells led to enhanced processing and presentation of the immunogenic peptides by DCs (Carrasco-Marín *et al.*, 1998). Hsp 70 might act as a chaperone for the unfolded polypeptides to help them regain a functional structure or to direct them to a degradation pathway (Feder and Hofmann, 1999). The scavenger receptors on DCs (e.g. LOX-1 and CD36) that take up oxidised antigens might also be involved in the uptake of the oxidised tumour cells (Delneste *et al.*, 2002; Marsche *et al.*, 2001; Marsche *et al.*, 2003). The precise nature of the

chemical reactions that are responsible is the subject of intensive investigation in this laboratory.

An attractive alternative to immunisation with antigen preloaded DC was to directly inject the antigen (in the absence of DC) into the host. This would eliminate the need for *in vitro* culture of the DC before administration. It was showed in Fig. 5.9 that oxidised B16.F10 cells alone were only able to elicit poor anti-tumour responses, and strong responses were seen when DCs were used. One possible reason for the poor tumour-immunity observed was that the oxidised B16.F10 were rapidly cleared from the blood stream after IV vaccination and this did not allow the accumulation of the oxidised antigens in the spleen cells and/or lymph nodes to be taken up by the resident DCs. On the other hand, DCs loaded with oxidised antigens would migrate and efficiently present the oxidised B16.F10 cells to the T cells in spleen and lymph nodes. Oxidised B16.F10 cells given alone via the subcutaneous route also induced a very poor anti-tumour response (data not shown). It would be interesting to determine whether multiple doses of the oxidised B16.F10 cells given alone at different time points in the vaccination regime would give a better immune response.

In this study, it was demonstrated that oxidised B16.F10 melanoma were potent immunogens for DCs to induce anti-tumour T cell responses. The results presented here and in chapters 3 and 4 strongly suggested that such DC vaccines could be useful in the treatment of cancer. Due to time constraint, this preliminary study is far from completed. Further optimisation of the vaccination protocol *in vivo* is needed – e.g. the dose and frequency of the administration of the DC vaccines, the breadth and duration of the elicited immune responses, and whether the primed T cells could

directly recognise and lyse non-oxidised autologous tumour cells. Results in chapter 4 strongly suggested that mature DCs were superior to immature DCs in priming an anti-tumour response. Therefore it would be of great interest to compare the immunogenicity of mature to immature mouse bone marrow-derived DCs in this C57BL/6 mouse melanoma model. As seen in Fig. 5.3, the level of DC maturation with LPS, MPL and IFN-γ was low compared to untreated DCs. This might be because the LPS and MPL used had lost their usual potency, or a longer treatment time with LPS and MPL (e.g. 48 h instead of 24 h used here) or a high dose was needed. The ultimate goal of any immunotherapy is the ability to generate CTL that will directly recognise and lyse tumour cells. Thus it is essential to set up a tumour protection assay to determine whether immunisation with autologous DCs loaded with oxidised tumour cells (i.e. oxidised B16.F10 in the C57BL/6 mice) will provide the immunised subjects protection against tumour growth and result in prolongation of their survival. Other methods to boost the anti-tumour responses, e.g. maturing DCs with clinical approved MPL or CD40 ligand before *in vivo* administration, or preventing Tregs activation with CTLA-4 antibodies or PD-1L, could be incorporated in the vaccination regime. Nevertheless, the results from this mouse melanoma model supported the use of oxidised allogenic tumour cell line as immunogens for DC-based immunotherapy of cancer. This might present a novel and straight forward method as such ''standardised'' cell lines would eliminate the need to obtain patient-derived tumour cells that are more difficult to obtain and inherently more heterogeneous.

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5.5. Conclusions

- B16.F10 melanoma cells highly expressed TRP-2 and were sensitivity to HOCl-oxidation killing *in vitro*.

- Immunisation of mice with bone-marrow derived DCs preloaded with oxidised B16.F10 cells via the intravenous, and not intraperitoneal or subcutaneous routes of immunisation induced potent anti-TRP-2 and anti-B16.F10 responses.

- HOCl-oxidised B16.F10 cells were superior immunogens for DCs to prime specific T cells *in vivo* compared to heat-killed or HCl-killed B16.F10 cells.

- DCs were required for the processing and presentation of oxdised B16.F10 *in vivo* to prime tumour-specific T cells.

Chapter 6

General Discussion

6.1. Introduction

Due to the importance of DCs in regulating the outcome of an immune response, considerable attention has been given in a clinical context to their potential use in cancer treatments. The research performed in this thesis addresses issues surrounding DC-based tumour immunotherapy on two different levels. Firstly, the development of a robust *in vitro* human cell culture system serves as a mean to investigate the important parameters that govern successful DC priming of potent anti-tumour T cell responses. Comparative studies done between the healthy volunteers and ovarian cancer patients in remission not only help to highlight the similarities but also the differences in the immune functions of these two different groups. Such information would be invaluable in the future design of DC vaccines. Secondly, using the information generated from the *in vitro* studies, an *in vivo* mouse model of melanoma is used to investigate the immunogenicity of such DCs vaccines. Factors that could influence the anti-tumour responses *in vivo* are being assessed. Finally, the impact of this work on the future development of DC-based tumour immunotherapy is discussed.

6.2. Defining the *in vitro* and *in vivo* parameters for DC-based immunotherapy

6.2.1. Choice of tumour antigen for loading dendritic cells

DC-based immunotherapy is a promising adjuvant therapy for treating cancers which are refractory to conventional chemotherapeutic approaches, and this technology is further assisted by the identification of shared and unique TAAs expressed by a variety of histologically different tumours. One major question is the choice of antigen for loading DCs. Examples of antigens used include peptides encoding HLA-restricted immunodominant epitopes recognised by either $CD8^+$ or $CD4^+$ T cells,

whole tumour cell lysates, tumour DNA or RNA, and tumour exosomes. The most popular and straight forward method is to load DCs with one or more HLA-restricted peptides. It is relatively easy to synthesize large quantities of clinical grade peptides, however only patients possessing the required HLA expression(s) are eligible. Moreover, the resulting immune responses are often limited to the epitope(s) used for immunisation, and the longevity of MHC-peptide complexes *in vivo* is unknown. In addition, the affinity of peptides for their various HLA molecules varies and this could affect their immunogenicity *in vivo* should competition occur between/amongst the peptides. Lastly, the phenomenon of epitope spreading is only observed in a very small number of patients after single or multiple peptide immunisation (Brossart *et al.*, 2000; Disis *et al.*, 2004). Some research groups have incorporated peptides encoding epitopes recognised by $CD4^+$ T helper cells to elicit a stronger overall immune response through providing cognate help to $CD8^+$ T cells. However, few authentic tumour antigen CD4 epitopes have been defined to date. Peptide-based trials have met with limited success and the issues previously mentioned still need to be addressed.

A promising alternative to peptides is whole tumour cells. Tumour cells express a whole array of TAAs that are characterised and uncharacterised, and this rich source of antigens contains epitopes of both $CD8^+$ CTLs and $CD4^+$ T helper cells. This is important as the parallel presentation of both MHC Class I and II restricted antigens would help to generate a stronger overall anti-tumour response and long term $CD8^+$ T cell memory via $CD4^+$ T cell help (Toes *et al.*, 1999; Zajac *et al.*, 1998). Also, it would greatly diminish the chance of tumour escape compared to using single epitope vaccines. In addition, the use of whole tumour cells eliminates the need to define, test

and select for immunodominant epitopes. The tumour cells could be autologous, i.e. obtained from the patients, or allogeneic. The major drawback for using autologous tumour cells is that they are only useful in single patient-tailored anti-tumour immunotherapies, and they pose problems of collection, processing, reproducibility and inter-patient variability. On the other hand, allogeneic cell lines that share one or even several of the TAAs as autologous tumour cells provide a simpler method of delivering antigens to DCs.

The SK-OV-3 ovarian carcinoma cell line used in this study highly expresses the two important ovarian TAAs, i.e. HER-2/neu and MUC1 (Fig. 3.1), found in a significant number of primary and metastatic ovarian cancers. The results in this thesis showed that priming T cells derived from both healthy volunteers and ovarian cancer patients with autologus DCs pulsed with oxidised SK-OV-3 generated $CD8^+$ and $CD4^+$ T cell responses directed against specific epitopes of HER-2/neu and MUC1. These results strongly suggested that it is unnecessary to match the HLA of the cell line (HLA-$A3^+$ and $A2^-$) to the patients/volunteers (most were HLA-$A2^+$) as the T cells will be primed to antigens presented in the context of the host MHC on host DCs. Another advantage is that it is relatively easy to propagate SK-OV-3 tumour cells to sufficient quantities for the purpose of DC-based immunotherapy. Moreover, the quality of the SK-OV-3 cells can be easily controlled through stringent laboratory procedures. However, a potential problem with using allogeneic SK-OV-3 cell line is that they might not share enough common TAAs to induce anti-tumour responses that could cross-react and reject all ovarian cancers. This question could only be answered properly in controlled clinical trials involving a large number of ovarian cancer patients, perhaps in different stages of the disease including patients who are already

in remission. Nevertheless, allogeneic cell lines have been used in clinical trials for treating patients with melanoma and other cancers (Höltl *et al.*, 2002; Palucka *et al.*, 2006; Salcedo *et al.*, 2006; Stift *et al.*, 2003; Vilella *et al.*, 2004). This highlights the potential of SK-OV-3 cell line as a source of antigens for DC-based immunotherapy of ovarian cancer. This idea is further supported by the *in vivo* melanoma study in C57BL/6 mice. Specific anti-tumour responses were elicited when the mice were immunised with bone marrow-derived mouse DCs preloaded oxidised B16.F10 melanoma (Fig. 5.4), highlighting the effectiveness of cell lines as a source of antigens for DC.

6.2.2. Methods for killing and enhancing the immunogenicity of antigens

Live tumour cells are poorly immunogenic and can possess an array of mechanisms, such as loss of HLA class I expression or deficiencies in antigen processing and presentation pathways, to evade the immune system. Moreover, live tumour cells can secrete soluble factors, such as VEGF, soluble FasL, IL-10 and TGF-β to suppress DC and T cell functions (see chapter 1). In this thesis, a strong oxidising agent – HOCl, has been used to induce rapid necrotic tumour cell death and at the same time enhancing their immunogenicity. In a physiological context, HOCl is one of the first lines of defense of the human immune system against microbial pathogens. It also enhances the immunogenicity of protein antigens by several folds *in vivo* and *ex vivo*.

In this thesis, it has been shown that treatment of SK-OV-3 tumour cells with 60 μM HOCl or more induced greater than 99% necrotic cell death (Fig. 3.2). The HOCl-oxidised and not live SK-OV-3 cells are efficiently taken up by DCs (Fig. 3.3) and showed enhanced immunogenicity and primed potent anti-tumour T cell responses

when compared to priming with SK-OV-3 cells made necrotic by non-oxidative means (i.e. heat-killed, HCl-killed, freeze-thawed, or x-ray irradiated) [see chapter 3 results and Fig. 3.4]. This effect is also demonstrated *in vivo* with the B16.F10 model (Fig. 5.8). Although the exact mechanism is outside the scope of this thesis, the improvement in antigen immunogenicity might be explained by three possible reasons. First, HOCl can quantitatively deaminate serine and convert its side chain into an aldehyde, and some studies have shown that tagging protein antigens with such aldehydes led to significant improvement in the responses directed against the antigens (Anderson *et al.*, 1997; Anderson *et al.*, 1999; Hazen *et al.*, 1996b). Second, oxidation of protein antigens might allow protein unfolding (e.g. by the reduction of disulphide bonds in the protein) and therefore enhance both processing by DCs and exposure of immunogenic peptides to specific T cells (Carrasco-Marín *et al.*, 1998). The heat-shock protein, such as Hsp 70, might also play a role in chaperoning polypeptides that unfolded during oxidative stress to help them regain a functional structure or by directing them to a degradation pathway (Feder and Hofmann, 1999). Third, scavenger receptor such as the lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) might be involved in the uptake of HOCl-oxidised SK-OV-3 tumour cells. LOX-1 is a type D scavenger receptor expressed on the surface of dendritic cells, macrophages, smooth muscle cells and platelets (Chen *et al.*, 2002). It binds to a broad range of ligands that include oxidised low-density lipids (OxLDL), hyphochlorite-modified HDL, advanced glycation end products (AGE), aged/apoptotic cells, activated platelets and bacteria (Jono *et al.*, 2002; Kakutani *et al.*, 2000; Marsche *et al.*, 2001; Moriwaki *et al.*, 1998; Oka *et al.*, 1998; Shimaoka *et al.*, 2001). LOX-1 has been implicated in antigen cross-presentation in DCs (Delneste *et al.*, 2002), therefore it is reasonable to postulate that it is involved in the uptake of

oxidised SK-OV-3 by DCs and facilitate cross-presentation of ovarian TAAs to $CD8^+$ T cells. CD36, a class B scavenger receptor expressed by macrophages and DCs for endocytic take up of OxLDL (Marsche *et al.*, 2003) and AGE-proteins (Ohgami *et al.*, 2001), might also play a role in the uptake of oxidised SK-OV-3. Preliminary studies with other forms of oxidation (e.g. by H_2O_2) can also improve protein immunogenicity, and it would be interesting to determine if there are any differences in the type of immune response obtained from priming with SK-OV-3 cells treated with HOCl and H_2O_2.

6.2.3. Improving the stimulatory capacity of dendritic cells via maturation

Some of the advantages of *in vitro* manipulation of DCs include the ability to control the quality of the DCs (i.e. maturation status) and their expression level of desired antigens and/or costimulatory molecules, the selection of specific DC subset(s), and the *in vivo* administration of the prepared DCs at anatomical sites of interest (e.g. lymph nodes or tumours). In chapter 5, it is demonstrated in the B16.F10 melanoma model that DCs are required in the immunisation protocol as oxidised B16.F10 cells alone could not prime any anti-tumour response (Fig. 5.9). This might be due to the rapid clearance of oxidised B16.F10 cells from the blood stream after IV vaccination and hence did not allow for their accumulation and uptake by the residential DCs in the spleen cells and/or lymph nodes. In contrast, DCs preloaded with oxidised B16.F10 could migrate and efficiently present the oxidised antigens to the T cells in spleen and lymph nodes.

The use of mature DCs is preferred to immature DCs as the latter have been shown to induce T cell tolerance and deletion, and leads to the development of Tregs (Brocker,

1999; Dhodapkar *et al.*, 2001; Jonuleit *et al.*, 2000; Steinman *et al.*, 2003). In chapter 3, it is demonstrated that oxidised SK-OV-3 tumour cells induced a partial activation of DCs by upregulating the maturation associated markers – CD83, CD86 and CD40 (Table 3.1) However, this DC maturation is sub-optimal compared to the TLR agonist LPS, and additional stimulation of DC might further enhance the response. Priming with these partially activated, oxidised SK-OV-3 loaded DCs stimulated a predominantly $CD8^+$ response via cross-presentation. In chapter 4, two DC maturation stimuli, i.e. agonistic CD40 antibody and MPL that have already been approved for use in clinics, are being assessed. Using DCs derived from ovarian cancer patients in remission, it is demonstrated that both agonistic CD40 antibody and MPL induced the upregulation of CD83, CD86, CD40 and DR expression on the DCs. When the T cells are primed with oxidised SK-OV-3 loaded DCs that are matured with either agonistic antibody or MPL, a significant improvement in the overall anti-tumour response is observed (Fig. 4.4). In addition, the magnitude of $CD4^+$ T cell responses is increased by at least 2-fold when such matured DCs are used (Fig. 4.5). Improved $CD4^+$ response is more dependent on full maturation of the DCs, and it might reflect the greater dependency of the $CD4^+$ T cell response on costimulatory activity by the APCs. The ability to elicit CD4 T cell response in this priming culture system is important as increasing evidence suggests that $CD8^+$ T cell responses primed in the absence of cognate help are impaired functionally in a variety of ways (Chamoto *et al.*, 2006; Nishimura *et al.*, 1999; Xiang *et al.*, 2005; Zhang *et al.*, 2007). Critically, $CD4^+$ T cell help is required for developing long term $CD8^+$ T cell memory and CTL function (Toes *et al.*, 1999; Zajac *et al.*, 1998). Hence using mature DCs to stimulate both potent $CD8^+$ and $CD4^+$ T cell responses is desirable.

Other maturation stimuli that have already been used in DC clinical trials and could also be evaluated in this priming culture system includes the cytokine cocktail containing TNF-α, IL-1β, Poly I:C, IFN-α, and IFN-γ (Mailliard *et al.*, 2004), which has been shown to induce potent CTL responses. This is preferred to the monocyte-conditioned media mimic that contains TNF-α, IL-1β, IL-6, and PGE_2 (Jonuleit *et al.*, 1997) as there are concerns about PGE_2 mediating Th 2 polarisation and promoting IL-10 secretion by DCs (Morelli and Thomson, 2003). Such cytokine cocktail-matured DCs are also more effective than immature DCs in expanding Tregs (Banerjee *et al.*, 2006). Another area of concern is the induction of IL-17 producing Th17 $CD4^+$ T cells that are found to be involved in host defense against pathogens as well as in autoimmunity (Weaver *et al.*, 2006). IL-6 together with TGF-β (that is frequently present at high level in the tumour microenvironment) that have been found to be necessary for Th17 cell differentiation (Mangan *et al.*, 2006). Other potential DC maturation stimuli such as the TLR agonists (e.g. CpG ODN, R848 and imiquimod) could be used on their own or in combinations to stimulate a more potent DC maturation and production of pro-inflammatory cytokines. The ligation of CD40 with recombinant CD40 ligand (French *et al.*, 2007; Rew *et al.*, 2005; Watanabe *et al.*, 2003) or with a drug-inducible CD40 expression system (Hanks *et al.*, 2005) is also a promising method of inducing DC maturation and enhancing their production of IL-12, and CTL priming capacity.

6.2.4. Avoiding autoimmunity and generating tumour-specific responses

A persistent concern in tumour immunotherapy is that the immune system will be activated to recognise and kill cells other than the tumour, hence causing autoimmune disease. This is particularly important with the use of whole cell immunogens as all

cells share large amounts of "common" housekeeping proteins, as well as expressing smaller numbers of "tissue-specific" proteins. It is essential that priming with oxidised SK-OV-3 only generated anti-tumour responses directed against TAAs on ovarian tumour cells, and not predominantly to self proteins that are expressed on normal cells for normal cellular metabolic functions. In this thesis, it is demonstrated that the T cells that responded to SK-OV-3 and its TAAs but did not respond to the melanoma line MEL-11 or to melanoma TAAs expressed on the MEL-11 cell line. Conversely, T cells immunised to MEL-11 melanoma cells did not respond to SK-OV-3 cells or to its TAAs. These observations applied to both healthy volunteers and ovarian cancer patients (chapters 3 and 4). Moreover, T cells specific to MEL-11 showed no recognition for ovarian tumour cells from autologous ascites (Fig. 4.9). Thus in this model, it is important that both tissue-type specific and TAA-specific responses have been generated in relation to at least two quite distinct tumour cell types. The risk of autoimmunity still needs to be further addressed as there remain some risks of cross-reactivity with normal (untransformed) epithelial tissues that share some TAAs, such as breast and normal peritoneum. In the B16.F10 melanoma study, the DC vaccines were well-tolerated and did not induce any anti-organ or tissue reactions in the animals immediately or up to 23 days. More detailed studies looking for immunopathological responses are needed but the potential of such DC-oxidised tumour cell vaccines inducing autoimmunity seems low.

Though it is of paramount importance to avoid generalised non-tumour specific auto-immunity, effective tumour therapy is also dependent on the partial breaking of self tolerance. This close relationship between tumour response and the development of autoimmune pathology has been demonstrated in a recent trial (Gogas *et al.*, 2006).

Two questions relating to ovarian cancer are being addressed in this study. First, it is important to determine whether the T cells of ovarian cancer patients that have been exposed to high levels of ovarian TAAs antigens *in vivo* over prolonged periods and yet are apparently tolerant to them, could still respond effectively to the oxidised SK-OV-3 antigen priming. Second, whether these patients' T cells stimulated in response to SK-OV-3 cross-priming actually respond to autologous tumour, which is an obvious requirement for any potential immunotherapeutic approach. In chapter 4, it has been demonstrated that priming with DCs preloaded with oxidised SK-OV-3 stimulated autologous T cells derived from ovarian cancer patients that could efficiently recognise and respond to specific epitopes of HER-2/neu and MUC1 on SK-OV-3. This observation is especially important as T cells from patients whose tumours overexpressed MUC1 and/or HER-2/neu also exhibited anti-MUC1 and HER-2/neu responses. In addition, two patients (patient 23 and 26) with T cells that were primed with autologous DCs preloaded with oxidised SK-OV-3 efficiently recognised autologous tumour cells from ascites without the need of APCs (Figs. 4.7 and 4.8). These results showed that despite exposure to high levels of ovarian TAAs *in vivo*, ovarian cancer patients' T cells are not tolerant to ovarian TAAs and can be primed with oxidised SK-OV-3 to recognise autologous ovarian tumour cells. These results therefore suggested a generic method to stimulate an anti-tumour T cell response which will be capable of targeting autologous tumour in patients with ovarian cancer.

6.3. Limitations of *in vitro* and animal studies

The *in vitro* studies on human cell cultures have helped to identify the important parameters, such as antigens for DCs and maturation state of DCs, needed for

generating potent anti-tumour responses. The breath of the responses could be broadened to test other ovarian TAAs such as NY-ESO-1 which is expressed on many ovarian carcinomas. However, there are some limitations with these *in vitro* studies. First, it is not possible to predict the toxicity or the potential of autoimmunity induction by these DC vaccines *in vivo*. Second, it is not possible to model the tumour microenvironment *in vivo*, which is extremely complex and involves the interplay of many different cell types (including fibroblast, B cells and macrophages). Therefore vaccines have to be tested *in vivo* to evaluate their efficacy. Preliminary studies done in a mouse B16.F10 melanoma model confirmed some of the findings of the *in vitro* studies. Though not totally representative of human cancers, the results in the melanoma mouse model suggested the potential use of oxidised whole cell line as a source of immunogens for DCs, and the DC vaccines were well-tolerated and did not seem to induce autoimmunity such as depigmentation of the coat-colour. Certain parameters are species-specific, and might work well in one but not in the other. These could only be realistically tested in human clinical trials. One example is the route of administration of DC vaccines and it might significantly influence the outcome of the immunotherapy. Another example is the doses and duration of effectiveness of the DC vaccines. Mice and humans would require different dose of the DC vaccines when taking the body mass into considerations. This is also true when administrating a prime-boost regime and the number of boosts given would also be different. The general health of the patients at the beginning of the DC vaccination could also influence the outcome of the treatment.

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6.4. Future work

To increase the potency of DC vaccines, it is envisaged that it be used in combination with chemotherapy, radiotherapy or with other immunointerventions. For example in this study, the use of mature DC preloaded with oxidised allogeneic tumour cells is a promising method of inducing T cells that can recognise both ovarian TAAs and ovarian tumour cells in ascites. There are several methods to induce DC maturation and two stimuli evaluated in this study, i.e. agonistic CD40 antibody and MPL, significantly enhanced both $CD4^+$ and $CD8^+$ T cell responses. Other promising DC maturation stimuli that are already in use in clinics and can be assessed in future studies include the cytokine cocktail mixture containing Poly I:C, CD40 ligand, and TLR agonists such as CpG ODN, R848 and imiquimod. The TLR agonists can be used in combinations to further improve DC activation.

To block negative regulatory interactions between DCs and T cells, immunointerventions such as depletion of Tregs, administration of anti-CTLA4 and/or anti-PD1 are being pursued in human clinical trials. Such interventions could be administrated *in vivo* together with the DC vaccines to enhance the latter's efficacy. It might also be helpful to genetically modify DCs to produce cytokines, such as GM-CSF or IL-2, or to express increased levels of costimulatory molecules so to enhance T cell priming. Also of great interest is the use of other oxidising agents, such as H_2O_2 in killing and enhancing the immunogenecity of tumour cells. It might be important to determine if there are any differences in the immune responses elicited by these different oxidising agents.

The ultimate goal of tumour immunotherapy is the direct lysis of autologous tumour cells by CTLs. Thus it would be imperative to test such ability in the primed T cells obtained from ovarian cancer patients using chromium release assay or other non-radioactive CTL assays. A multi-colour flow cytometry assay could also be performed to assess the cytokine profiles of the primed T cells. A major limitation of this study is the small number of ovarian cancer patients being recruited (n=27), and amongst them only two had autologous ascites available. A larger patient sample size will give a clearer picture of the overall efficacy of priming T cells with autologous DCs loaded with oxidised SK-OV-3 cells. In addition to evaluating the responses in cancer patients in remission, patients with other stages of ovarian cancer (in particular Stage I patients with small tumour burdens) could also be studied.

In the B16.F10 melanoma study, it would be essential to conduct a tumour protection assay to further determine the crucial parameters governing effective anti-tumour response. In this assay, the tumour growth and survival of the mice would be monitored following DC vaccine administration. It is also of great interest to assess the immunogenicity of mature DCs preloaded with oxidised B16.F10 in the C57BL/6 melanoma model. In addition, a prime-boost protocol could be developed to determine the optimal amount of DC vaccines and time for administration. As a mouse ovarian tumour model is now available, it would be especially useful to test the DC-oxidised SK-OV-3 vaccines in the transgenic HLA-A2 expressing mouse ovarian tumour model. The responses to oxidised SK-OV-3 and to HLA-A2 restricted epitopes of MUC1, HER-2/neu and other ovarian TAAs (such as NY-ESO-1) could be tracked in this model. CTL and tumour protection assays could also be performed.

Chapter 7

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